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Investigating the Balance Between Estrogen Receptor Mediated Cell Proliferation and Genomic Surveillance

Margarita Brown
University of Massachusetts Amherst

Investigating the Balance Between Estrogen Receptor Mediated Cell Proliferation and Genomic Surveillance

A Thesis Presented

by

MARGARITA M. BROWN

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MARGARITA M. BROWN

Approved as to style and content by:

Karen A. Dunphy, Chair

Sallie W. Schneider, Member

Laura N. Vandenberg, Member

Rafael A. Fissore, Department Head
Veterinary and Animal Sciences Department

DEDICATION

To my mentors and all that my find this work useful.

ACKNOWLEDGEMENTS

I would like to thank my advisors Dr. Jerry, Dr. Dunphy and Amy Roberts for their tireless support of my goals. Thank you to my lab mates for reminding me to have fun. Thank you to my family for keeping me focused on what is important.

ABSTRACT

INVESTIGATING THE BALANCE BETWEEN ESTROGEN RECEPTOR MEDIATED CELL PROLIFERATION AND GENOMIC SURVEILLANCE

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MARGARITA M. BROWN, B.A., MOUNT HOLYOKE COLLEGE

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Dr. Karen A. Dunphy

Breast cancer is a leading cause of cancer in women and the second leading cause of cancer death. Lifetime exposure to estrogen contributes to this risk but high dose estrogen has been used to induce apoptosis as treatment for breast cancer. These opposing tumorigenic and anti-tumorigenic effects of estrogen may be regulated differently by the two Estrogen Receptors (ER), Estrogen Receptor alpha (ERα) and Estrogen Receptor beta (ERβ). Although the receptors share a 96% homology in their DNA binding domain, they are unique in the ligand-binding domain with 53% amino acid homology. Previous studies have shown that ERα drives cell proliferation in the mammary gland. We propose that ERβ mediates genomic surveillance in the mammary gland to restrict proliferation. To test this hypothesis we first characterized each of our reference breast cancer cell lines to determine the ERα and ERβstatus. We found that ERβ transcript and protein are expressed in some breast cancer cell lines that are considered to be “triple-negative” (HCC1937 and MDA MB 231). Using specific

ER agonists, we were able to demonstrate that amphiregulin, a secreted protein and a marker of ERα activation, is upregulated by ERα agonists in a dose dependent manner in cell lines that have ERα (T47D & MCF7). ERα agonists do not enhance AREG expression in cell lines that primarily expresses ERβ (HCC1937). Instead, CEBPd, a tumor suppressor, is expressed at high levels in this cell line. In conclusion, targeting ERβ has the potential to selectively activate tumor suppressor pathways without stimulating proliferation and may provide a treatment option for patients for whom inhibition of ERα is not an option.

TABLE OF CONTENTS

Page

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

The Dual Roles of Estrogen

Globally, the most common cancer in women is breast cancer. In the United States, the probability of a woman developing breast cancer in her lifespan is 1 in 8 (Siegel, 2015). Although many factors play a role in the occurrence of cancer, including a woman's genetics and lifestyle choices, the greatest contribution to breast cancer risk in a lifetime stems from chronic exposure to estrogen (K. N. Anderson, Schwab, & Martinez, 2014).

Estrogen has duplicitous effects in the breast, in that it both promotes and reduces breast cancer risk (Folkerd E, 2013). Early menarche, late menopause and estrogen exposure post-menopause promote breast cancer risk, while an early full term pregnancy reduces that risk by 50% (MacMahon et al., 1970). *In vivo* studies in rats using estrogen and progesterone to mimic levels during pregnancy showed a reduction in the incidence of mammary tumors (Cabanes et al., 2004; Rajkumar et al., 2001; Sivaraman et al., 1998). Also, parous and hormone treated mice had a greater apoptotic response to ionizing radiation compared to nulliparous mice (Dunphy KA, 2008). High doses of estrogen have also been used successfully to treat breast cancer in postmenopausal women by increasing apoptosis and decreasing tumor size (G. L. Anderson et al., 2012;

Ellis et al., 2009; Haddow, Watkinson, Paterson, & Koller, 1944). These dual roles indicate a potential balance between estrogen-mediated pathways that regulate proliferation versus genomic surveillance facilitated by cell cycle arrest and apoptosis. The opposition of effects between activation and abatement of proliferation might be regulated by different estrogen receptors in order to maintain the homeostatic balance within the mammary epithelium.

Estrogen signals via two receptors, Estrogen receptor alpha (ERα) and Estrogen receptor beta (ERβ), to regulate gene transcription (Gruber, Gruber, Gruber, Wieser, & Huber, 2004). Two ligand bound estrogen receptors located in the nucleus dimerize and bind to an estrogen response element (ERE) on DNA. Recruitment of co-activators and co-repressors interacting with the DNA bound estrogen receptor-ligand complex assist in transcription regulation in tissues (Kurebayashi et al., 2000). Estrogen receptors can homodimerize forming an ERα-ERα or ERβ-ERβ complex; or they can heterodimerize as ERα-ERβ (Ogawa et al., 1998). Dimerization of the different homodimers and heterodimers could elicit different effects in the regulation and transcription of genes (Monroe et al., 2005).

Two separate genes, ESR1 and ESR2, encode ERα and ERβ respectively. Estrogen receptors have four functional domains and a hinge region (Figure 1.1). The N-terminal domain (A/B) is regulatory, and contains an activation function region (AF-1) that coordinates the receptors interaction with transcription factors (Metzger, Ali, Bornert, & Chambon, 1995) .The DNA binding

domain (C/D) is highly conserved between both ERα and ERβ and the receptors share a 96% conserved region that binds to the hormone response element on DNA. The hinge region (D) gives the receptor flexibility between the DNA binding domain and ligand binding domain. The E/F domain contains both the ligand binding pocket and the AF-2 region that directly contacts coactivator peptides. The function of the F domain on the C-terminal is still being investigated. Though the two ERs are similar in their DNA binding region, they are distinct in their ligand-binding domain with only 53% homology (Reese et al., 2014). Because the DNA binding domain is highly conserved (96%) between ERα and ERβ, both receptors have similar affinity for the ERE (Klinge, 2001). However, because the ligand-binding domain is distinct (53%) between ERα and ERβ, this region confers ligand specificity to each receptor that can be used to stimulate one receptor and not the other.

ERα and ERβ have distinct tissue expression patterns in different tissues of the body. Regionally, ERα is expressed in the pituitary gland and the uterus while ERβ is predominately found in the lungs and the bladder. Both receptors are expressed in the normal mammary gland (Mueller & Korach, 2001). ERα is expressed in 15-30% of luminal epithelial cells while ERβ is expressed in luminal epithelial, myoepithelial, fibroblasts and adipocytes (Figure 1.2)(Anderson, 2002). Interestingly, ER positive status is lost during cancer progression and in many breast cancer cell lines (Park P 2001, Shaaban AM 2003, Roger P 2001, Bardin 2004). The ER positive phenotype is also progressively lost in primary cell culture

of human breast cancer tumors (V. Speirs, 1998). Confirming ERα and ERβ status in ER positive cell lines: T47D, MCF-7, ZR 75-1, 76N TERT and in ER negative cell lines: MDA 231 and HCC1937, as well as assessment of an ERβ inducible cell line: MCF-7 –tetracycline-repressible ERβ (MTO ERβ), will cumulatively be powerful *in vitro* tools for the assessment of growth responses based on ER ratios (Figure 1.3).

Both receptors bind 17β-estradiol (E2), the endogenous ligand, with an equal affinity (Figure 1.4). Estrogen receptor ligands with specific affinities for ERα or ERβ exist. All estrogen receptor agonists are measured in relation to the competitive binding between E2 and the competitor. The ERα specific ligand, Propyl-pyrazole-triol (PPT) shows >410-fold selectivity for ERα over ERβ. Whereas 7-Ethenyl-2- (3-fluoro-4-hydroxyphenyl)-5-benzoxazolol (ERB041) and Diarylpropionitrile (DPN) display >200-fold and 70-fold selectivity for ERβ over ERα, respectively (Kuiper et al., 1997). Using ER specific ligands to bind to either ERα or ERβ will give us the ability to examine the distinct transcriptional properties of the receptors in the context of the different cell lines.

Estrogen receptor selective agonists have been used in the past to study both the balance of ERα and ERβ in cell lines as well as the expression of their target genes. One study used a tetracycline inducible ERβ variant of the human osteosarcoma line (U2OS) to measure how the ratio of ERα and ERβ effected cell proliferation induced by E2, the ERα agonists PPT, and the ERβ agonist DPN. They found that increasing the expression of ERβ and adding the ERβ

agonist DPN corresponded to curbed cell proliferation when compared to cells treated with E2. Also, they found that the addition of the ERα agonist PPT to the cells, regardless of ER expression, would stimulate cell proliferation (Sotoca et al., 2008). Another study used the breast cancer cell line MCF-7 to investigate the transcriptional profiles of ERα and ERβ stimulation. MCF-7 cells that were treated with E2 were shown to up regulate genes that signal for cellular proliferation including Amphiregulin (AREG) while simultaneously down regulating Transforming growth factor beta (TGFβ), a cytokine that restricts cell growth (Frasor et al., 2003). An additional study found that ERβ specific stimulation by DPN increases the PTEN tumor suppressor in MCF-7 and T47D breast cancer cell lines (Lindberg, Helguero, Omoto, Gustafsson, & Haldosen, 2011). This indicates that there are specific estrogen receptor roles in the regulation of cell proliferation.

Breast cancer is grouped by receptor status through immunohistological (IHC) characterization. Broadly, breast tissues stain positively for estrogen receptor alpha (ERα+) and progesterone receptor (PR+) are classified as "Receptor Positive" or "Luminal Type" breast cancer. Currently, therapeutics that antagonize the estrogen receptor exist and prognosis is generally good. Targeted therapeutics are also available for breast cancers that stain positively for HER-2/neu (HER-2+). Unfortunately, 15% of breast cancers do not stain for any receptor and are classified as triple negative breast cancer (TNBC)(Chacón & Costanzo, 2010). TNBC is more likely to reoccur and has the poorest prognosis

because there are no targeted treatments available. Though both ERα and ERβ are expressed in the mammary gland (Kuiper GG, 2007), only ERα is routinely tested for (Allred, 2010). However, the ER content of breast tumors that were categorized as triple negative (TNBC) has been investigated and it was determined that up to 25% of TNBCs expressed ERβ (Reese et al., 2014). Therefore, ERβ targeted therapy is a potential option to elicit a genomic surveillance response and cell cycle arrest in TNBC.

The dual roles for estrogens and their contribution to genomic surveillance and proliferation have been investigated previously in the Jerry lab. BALB/c-Trp53$^{+/+}$ mice were ovariectomized and allowed to recover and clear endogenous hormones for one week. They received daily intraperitoneal injections for 4 days with vehicle, E2 (2ug), progesterone (P; 200ug) and an E2+P combination and were subjected to 5Gy ionizing radiation prior to tissue harvest (Becker et al., 2005). Nuclear protein expression of radiation-induced p21, a cell cycle inhibitor, was upregulated to the greatest extent in the E2 or E2+P treatment groups compared to vehicle and P alone. Furthermore, treatment with E2+P + ICI 182,780 (an estrogen receptor inhibitor) mirrored the lower radiation-induced p21 response to progesterone alone. E2+P +mifepristone (an inhibitor of progesterone receptor) retained a strong p21 response to ionizing radiation. This indicated that the estrogen receptor was necessary to fully potentiate radiation-induced genomic surveillance cell cycle arrest pathways.

To examine the contribution of the two estrogen receptors, ERα and ERβ, in terms of proliferation and genomic surveillance responses, ovariectomized BALB/c mice were implanted with silastic capsules containing cellulose (Control) or cellulose with Progesterone (20mg) together with E2 (50ug), or one of the ER selective agonists: for ERα - PPT (400ug) and for ERβ - DPN (400ug) (Erick Roman-Perez, unpublished. After 3 days, mice were subjected to ionizing radiation. Apoptotic responses were increased to a similar extent by all hormones when compared to control (Figure 1.5a). However, the only E2+P and PPT+P increased proliferation as determined by BrdU incorporation and amphiregulin expression (Figure 1.5b). The ERβ agonist, DPN failed to activate proliferation. Roman-Perez *et al* concluded that the ERβ specific agonists (DPN) could activate genomic surveillance via induction of apoptosis in response to radiation without inducing proliferation.

Hypothesis and Rationale

Estrogen bound to an Estrogen Receptor can stimulate proliferation and to enhance genomic surveillance to prevent the unwanted proliferation of damaged cells. These dual roles are central to the paradox of estrogen- the ability of estrogen to both promote and inhibit breast cancers. This is possibly due to the ratio of the two estrogen receptors, alpha and beta, and their ability to balance estrogen-mediated proliferation with genomic surveillance to activate apoptosis or cell cycle arrest. The two estrogen receptors are 96% homologous in their DNA binding domain, meaning that they bind to similar estrogen response

elements on DNA, but are distinctive in their ligand-binding domain with 53% amino acid homology. This means that the two different estrogen receptors can be selectively targeted with selective estrogen receptor agonists.

Specific estrogen receptor agonists have been used in the past to compare Estrogen Receptor responses *in vitro* using human breast cancer cell lines and in *vivo* using mouse models. Preliminary data in mice show that radiation induced apoptosis is increased with estrogen receptor agonists (Roman-Perez, unpublished). Only the ERβ specific agonist could induce apoptosis without activating proliferation.

Hypothesis: Specific activation of ERβ in the human mammary gland selectively activates genes that mediate genomic surveillance without stimulating proliferation.

Several Estrogen Receptor targets are of interest to breast cancer research because of their association with cell proliferation or genomic surveillance (Figure 1.6). The ERα associated protein amphiregulin (AREG) is of interest because it is an effector of estrogen signals and it works in a paracrine fashion to promote growth in neighboring epithelial cells (Peterson et al., 2015). A second target of interest is the Progesterone Receptor (PR) which is positively regulated by ERα ligand-dependent activation (Lin et al., 2004).

ERβ mediated growth restriction may occur by regulating the expression of cytokines and transcription factors. One cytokine of interest that may be modulated by ERβ is transforming growth factor beta 2 (TGFβ2). TGFβ2 is a

estrogen stimulated growth repressor that has been suggested to be regulated by ERβ in rat prostates (Itoh, Patel, Cupp, & Skinner, 1998). The transcription factor CCAAT/Enhancer Binding Protein (CEBPD) is also of interest because it is expressed in mammary luminal epithelial cells during involution and co-regulates pro-apoptotic genes (Thangaraju et al., 2005; Yu, Si, Zhang, & DeWille, 2010). Investigating the targets of Estrogen Receptors may help elucidate the molecular events that govern breast cancer cell proliferation and genomic surveillance.

Targeting a hormone receptor has been a successful therapy for breast cancer for patients who test positive for ERα, PR or Her2. Current targeted therapies for Estrogen Receptor positive breast cancer aim to inhibit the proliferative effects of ERα. There are no targeted therapies for Triple Negative Breast Cancer (TNBCs), which lack ERα, progesterone receptor (PGR) and human epidermal growth factor receptor 2 (HER2). TNBCs have poor prognosis. However, some TNBCs express ERβ. Targeting ERβ has the potential to selectively activate surveillance pathways without stimulating proliferation and may provide a treatment option for patients for whom inhibition of ERα is not an option (both ERα+ non-responders and TNBCs). Further, the expression of a higher ratio of ERβ to ERα may be important to an increased disease-free and overall survival in patients with triple negative breast cancer (Honma et al., 2008).

Objectives

Objective 1. Characterize the ratios of ERα and ERβ in cell lines by gene and protein expression.

Luminal type: MCF-7, T47D, ZR75-1, MTO ERβ

Triple negative breast cancer: MDA-MB-231, HCC 1937

"Normal": 76N Tert

Objective 2: Compare responses to different estrogen receptor agonists in breast cancer cell lines *in vitro*.

- Cell proliferation measured against cell death
- Gene expression of proliferation genes vs. surveillance genes

Objective 3: Conduct an *in vivo* experiment using the βERKO mouse model to explicate the histologic and gene expression profiles of acute agonist exposure in mouse mammary tissue for proliferation and markers of surveillance.

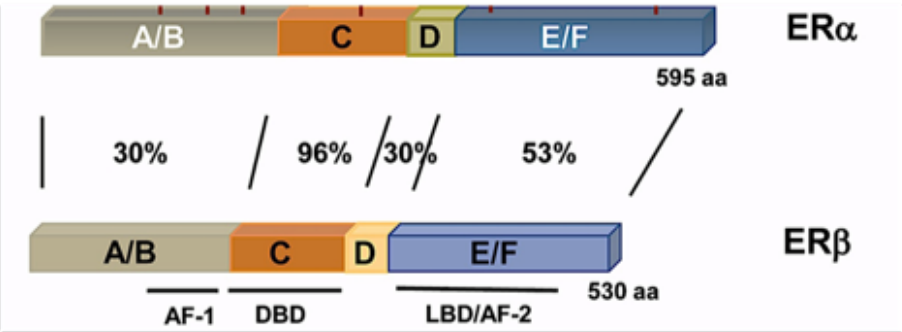


Figure 1.1 Structure and Homology of the Estrogen Receptors. Estrogen Receptor alpha (ERα) and Estrogen Receptor beta (ERβ). ERα and ERβ share a 96% amino acid homology in their DNA binding domain (DBD). They are distinctive in their activation function domain (AF-1, 30% homology), hinge domain (D; 30% homology) and in their ligand-binding domain (LBD/AF-2; E/F; 53% homology). The differences in these domains confer both ligand binding specificity as well as distinct transactivation functions to the receptors, allowing for the recruitment of transcription factors that mediate cell proliferation and genomic surveillance.

Adapted fron Roman-Blas, J. A., Casteneda , S., Largo, R., & Herrero-Beaumont, G. (2009). Osteoarthritis associated with estrogen deficiency. Arthritis Res Ther, 11(5), 241.

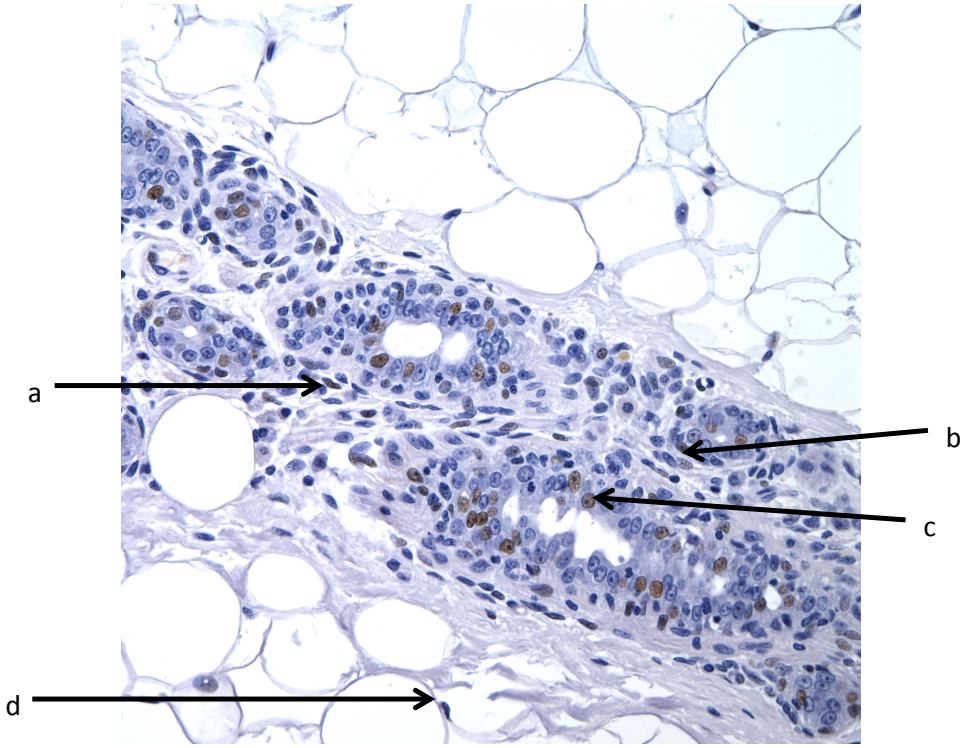


Figure 1.2 Expression of ERβ in human mammary gland.
Immunohistochemistry (IHC) of a human mammary gland using 14C8 ERβ antibody shows that ERβ is expressed in fibroblasts (a), myoepithelial cells (b), luminal cells (c) and adipocytes (d).

Photo by Karen Dunphy

Cell Line	ER	PR	Her2	P53
T47D	+	+	+	leu to phe
MCF-7	+	+	+	wt
MDA MB 231	-	-	-	arg to lys
MCF-7 MTO ERB Repressed	+	+	+	wt
HCC1937	-	-	-	mutant (stop)
ZR75-1	+	+	+	wt
76N tert	+	+	n/a	wt
MCF-7 MTO ERB Expressed	+	+	+	wt
HeLa	-	+	-	wt

Figure 1.3 Table of Breast Cancer Cell Line Characteristics.

Soussi, Thierry. "Handbook of p53 mutation in cell lines." Version 1, no. 07 (2007): 2007.

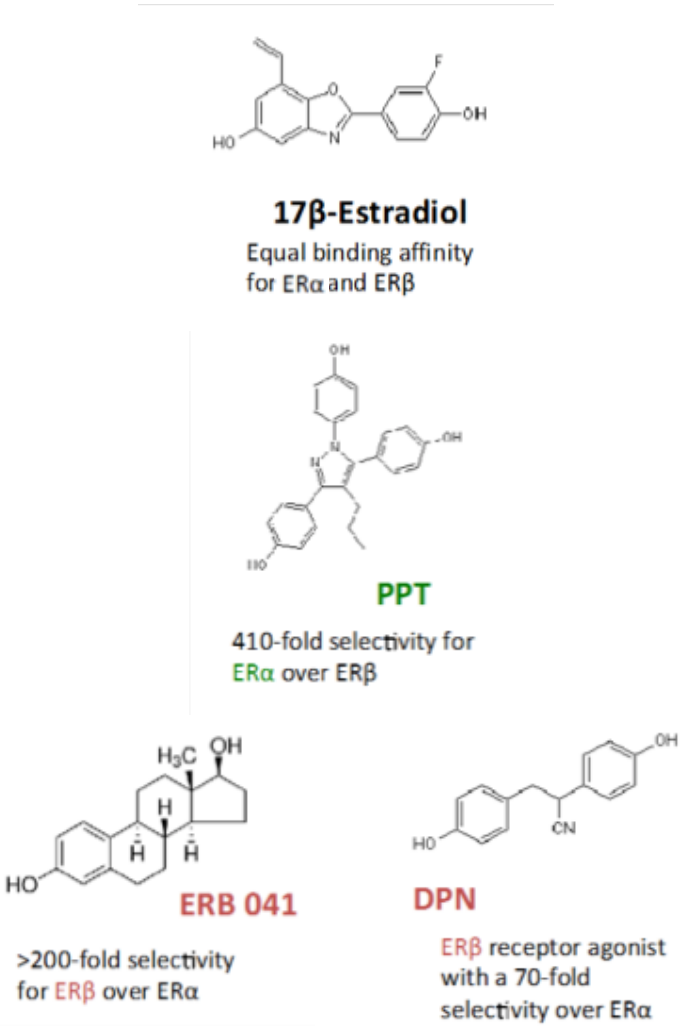
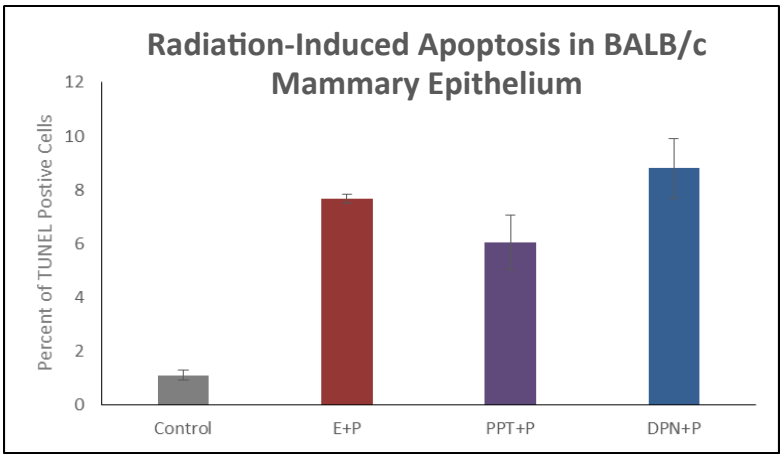


Figure 1.4 Estrogen Receptor Agonists. Endogenous and exogenous ligands for the estrogen receptor and their relative binding affinities for the different receptors relative to 17β-estradiol.

a.



b

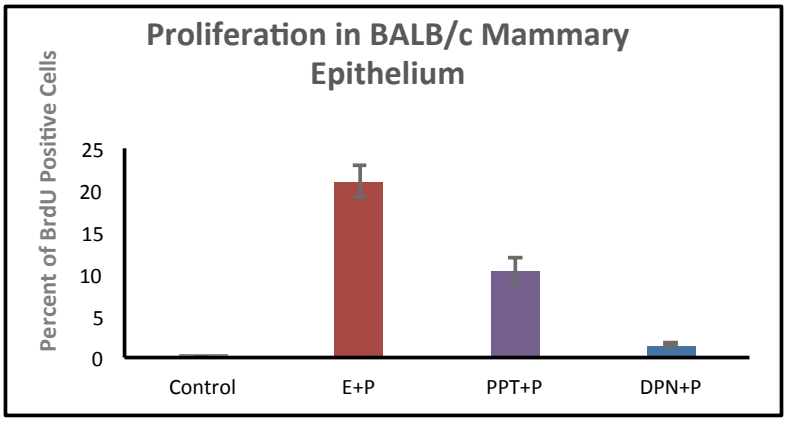


Figure 1.5 Proliferation and Apoptosis in the BALB/c Mouse Mammary Gland in Response to Agonists. Estrogen receptor agonists 17β-estradiol (E), 4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT) and Diarylpropionitrile (DPN) increase radiation induced apoptosis response in BALBc mice when combined with progesterone (P) to mimic the protective effect of parity (a), but only 17β-estradiol and PPT induce proliferation (b), DPN a potent Estrogen Receptor β agonist failed to induce proliferation.

Erick Roman-Perez, et al. unpublished

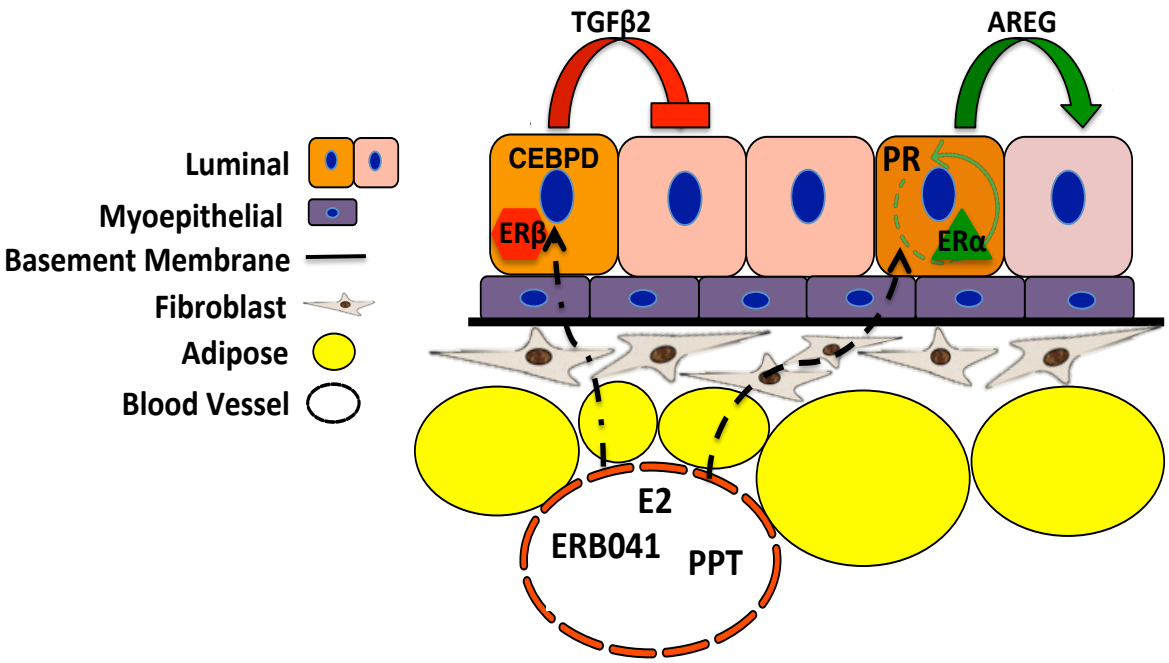


Figure 1.6 Model depicting hypothesis whereby estradiol stimulates proliferation or genomic surveillance by regulating gene expression including cytokines.

CHAPTER 2

CHARACTERIZATION OF THE RATIOS OF ERα AND ERβ IN CELL LINES BY GENE AND PROTEIN EXPRESSION

Introduction/Rationale

Examining the expression of ERα and ERβ across cell lines is important because there are conflicts with regard to the ER status of human breast cancer cell lines in the literature (Figure 2.1 a&b). For example, the MCF-7 cell line is regarded as a model of an exclusively ERα positive cell line (Felzen et al., 2015; Hsieh, Santell, Haslam, & Helferich, 1998). Yet some report that the MCF-7cell line also expressed ERβ (Tong et al., 2002; Vladusic, Hornby, Guerra-Vladusic, Lakins, & Lupu, 2000). Further, the MDA MB 231 cell line is used as a model for ER negative breast cancers (Price, Polyzos, Dan Zhang, & Daniels, 1990) and published data from three sources, using both RT-PCR and western blot assays, find that the MDA MB 231 cell line, are, in fact, ERα negative (Kao et al., 2009; Subik et al., 2010; Tong et al., 2002). However, other published reports demonstrate that MDA MB 231 is both ERα (Ford, Al-Bader, Al-Ayadhi, & Francis, 2011) and ERβ positive (Ford et al., 2011; Tong et al., 2002). Establishing ER status in our reference cell lines is necessary for the examination of the ER agonist responses. The selected human cell lines represent “normal” breast epithelium (76N Tert), luminal type breast cancers (MCF-7, T47D, ZR75-1, MTO ERβ) and TNBCs (MDA-MB-231, HCC 1937). To compare the ratios of the

receptors, expression of ESR1 and ESR2, the genes for ERα and ERβ respectively, will be assessed using qPCR and protein will be evaluated with western blots. Primers for qPCR amplification are included in Figure 2.2.

Detection of ERα expression was accomplished using the historically validated antibody SC-542 (MC-20), a polyclonal rabbit ERα antibody from Santa Cruz (Karen A. Dunphy et al., 2013). Immunohistochemical and western blot detection of ERβ has been unreliable in the past because the available antibodies are inconsistent (Skliris et al., 2002). Recently, a paper from the Mercurio lab at UMass Medical School investigated how the loss of ERβ effected chronic inflammation in human prostates (Mak, Li, Samanta, & Mercurio, 2015). They used the GeneTex ERβ antibody GTX112927 to observe ERβ expression in prostate cell lines. This rabbit polyclonal antibody was made against a recombinant peptide sequence within the center region of human ERβ (Figure 2.3). Additionally, because western blot detection and specificity of ERβ antibodies have been inconsistent in the past, we will also consider the primary antibodies GTX70174 (GeneTex) mouse monoclonal antibody, clone 14C8, developed against aa 1-153 of human ERβ, PA1-311 (Thermo Scientific) rabbit polyclonal, immunogen aa 55-70 of rat ERβ and PA1-310B (Thermo Scientific) rabbit polyclonal, immunogen aa 467-485 as potential options for assessing ERβ expression in human cell lines (Figure 2.3).

There are five described isoforms of ERβ, of which only ERβ1 contains a functional ligand-binding domain capable of binding hormone (Leung, Mak,

Hassan, & Ho, 2006). Three of the antibodies (GTX112927, GTX70174 and PA1-311) will bind all isoforms of ERβ while only PA1-310B will bind to the unique intact C-terminal end of ERβ1 (Figure 2.3). ERβ2, 4 and 5 do not have the ability to increase gene activation on their own, they must dimerize with ERβ1. It is therefore useful to include primary antibodies that are able to recognize functional ERβ1 in this study.

The efficacy and specificity of the ERβ antibodies will be assessed using the inducible ERβ cell line MCF-7 Tet-Off (MTO ERβ) as a positive control. MTO ERβ is a human breast cancer derived cell line stably transfected with tetracycline repressible estrogen receptor beta. The cells express a tetracycline regulated Tet-Off transactivator. Inducible expression of ERβ occurs when doxycycline (dox) is withdrawn from the culture medium. Conversely, in the presence of dox, ERβ expression is repressed. The inducible MTO ERβ is a good positive control for ERβ because we can regulate ERβ expression and have already determined the doses of doxycycline for efficient repression of ERβ (Figure 2.4). As a negative control, the human cervical adenocarcinoma derived cell line HeLa, was used, as it is negative for both ERα and ERβ (Holliday & Speirs, 2011).

Materials and Methods

Cell Culture: T47D and MDA MB 231 cells were kindly donated by Dr. Sallie Schneider. MCF-7 tet off cells were provided by Dr. L. Hodges-Gallagher (Hodges-Gallagher et. al., 2007); ZR 75-1 from Dr. Charles Perou, 76N tert cells

from Dr. Vimla Band, Hela cells from Dr. Rong Shao. HCC1937 were purchased from ATCC. All cells were cultured at 37°C and 5% CO2. Each Cell line was expanded in their standard Dulbecco's Modified Eagle's Medium/Nutrient mixture F1-12 Ham (DMEM:F12, Sigma-Aldrich; St. Louis, MO) with 15 mM $NaHCO_3$ and 25 mM Hepes. Cells were plated in triplicate, either at 500,000 cells/well or 1,000,000 cells/well in a six well plate (Cell Treat; Shirley, MA) at 50-60% confluence in order to achieve optimum 75-80% cell density after 24hrs of incubation. The cells were harvested for mRNA (three replicates) or lysates (three replicates). To repress the expression of ERβ in the MCF-7 tet off cells, cells were maintained in 50 ng/ml Doxycycline (Dox)(Sigma-Aldrich; St. Louis, MO). To repress ERα, cells were treated with 10nm ICI 182,780, 10nm E2 or both.

Primer Design and Efficiency Determination: Oligonucleotide primers were designed with software from qPrimerDepot (Dr. Wenwu Cui PhD, National Institutes of Health) and analyzed using the Integrated DNA Technologies (IDT) OligoAnalyzer software (Redwood City, CA), with sequences available from the PubMed database. The primer pairs were selected using the following criteria: the cDNA amplicon will be about 100 base pairs, primers are designed to flank intron-exon borders or primers that will anneal at a splice junction to distinguish genomic DNA from cDNA, primers will have similar annealing temps at or around 60°C, with a G/C content of 20-70% and low or no self-complementarity sequence. Table 2.2 lists the genes as well as the forward and reverse

sequence. Primer efficiency was determined by measurement of the gradient of a standard curve. The log of the target concentration was plotted against the quantitation cycle. Efficiencies at or close to 100% were accepted.

mRNA Isolation and RT-qPCR: RNA was isolated from cell culture using 1 ml TRIzol™ reagent (Life Technologies, Carlsbad, CA) per well (6 well plate), according to the manufacturer's instructions. Complementary DNA was synthesized using 1 ug of RNA, $d(T)_{23}VN$(50µM) and ProtoScript II Reverse Transcriptase (New England BioLabs Inc., Ipswich, MA). qPCRs were carried out in a MJ Research PTC-100 thermal cycler (Bio-Rad; Hercules, California). Quantification of each cDNA was achieved using SYBR Green Master Mix Reagent (Applied Biosystems; Foster City, CA) in duplicate. Gene expression is shown relative to T47D cells treated with 10nM ICI as an inter run calibrator (IRC). Relative quantification was performed using a comparative CT method. Values shown are relative to the IRC. Ratios of Estrogen Receptors were made relative to T47D cells.

Western Blotting: Whole cell extracts were lysed using 300µl/well (6 well plate) of ice-cold RIPA lyses buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 1 % Triton X-100; 1 % Sodium deoxycolate; 0.1 % SDS; 1 % protease inhibitors (P8340 Sigma-Aldrich; St. Louis, Mo), 1% phosphatase inhibitor #2(P5726, Sigma-Aldrich; St. Louis, MO), 1% phosphatase inhibitor #3 (P0044 Sigma-Aldrich; St. Louis, Mo) (Sigma-Aldrich; St. Louis, MO). Following centrifugation of the homogenate for 15 min at 13,000 rpm at 4 °C, the

supernatant containing the protein was removed from the cellular debris and quantified by the Bradford method. Equal amounts of protein (20 µg) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) through 10 % acrylamide under reducing conditions and then blotted onto Hydrophobic Polyvinylidene Fluoride (PVDF) membrane (Immobilon-P; Millipore, Watford, United Kingdom). The blot was blocked with a 5 % non-fat dry milk in TBST (10 mM tris-HCl pH 7.5, 150 mM NaCl, 0.1 % tween-20) for 60 min and subsequently incubated overnight at 4 °C with polyclonal anti- ERα (Santa Cruz Biotechnology Inc, MC-20: sc-542; 1:1000), anti-ERβ (Thermo Scientific PA1-311B; 1:2000 PA1-310B; 1:1000; GeneTex GTX70174; 1:1500), and anti-β actin (Sigma A1978; 1:5000). After washing in TBST, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h. The bands were visualized by enhanced chemiluminescence (100 mM Tris/HCl pH 8.5, 250 mM luminol (Sigma,Aldrich; St. Louis, MO), 90 mM p-coumaric acid (Sigma-Aldrich; St. Louis, MO), 30% hydrogen peroxide(Sigma-Aldrich; St. Louis, MO), imaged using G:BOX (Synoptics Ltd; Frederick, MD) and quantified using GeneTools analysis software from Syngene (Synoptics Ltd; Frederick, MD). The expected molecular weight for the protein product for the western blot is 66 kDa for ERα, 59 kDa for ERβ and 42 kDa for the β actin loading control. All cell lines were run in two independent experiments, each cell line band was quantified using average luminescence and compared relative to the T47D cell line.

Ratio of ESR2: ESR1 and ERβ: ERα: Expression was set relative to T47D cells. Assuming ESR2: ESR1 and ERβ: ERα is 1:1 in T47D cells, the ratio for each cell line were determined. These were calculated by comparing relative values. For example ESR2: ESR1 in MCF-7 parental cells: ESR2 0.12/ ESR1 1.13 = 0.106. Likewise for ERβ: ERα, this was calculated by comparing relative values in MCF-7 parental cells: ERβ 1.29/ ERα 0.46 = 2.8.

Statistics: A two sided students t-test was used to determine differences relative to T47D cells. A p-value <0.05 (*) was considered significant and <0.01 (**) considered highly significant. Error bars indicate SEM.

Results

ESR1 and ESR2 have a distinct expression across cell lines

ESR1 and ESR2 expression was evaluated using cDNA generated from the RNA harvested from the individually cultured cell lines (Figure 2.5 a&b). Expression is relative to T47D cells because they were expected to express both ERα and ERβ (Ford et al., 2011). ESR1, encoding Estrogen Receptor alpha, was expressed to a similar level in T47D, MCF-7 parental cell line as well as the MTO ERβ cell line, regardless of whether ERβ was expressed or repressed. The cell line ZR 75-1 and the triple negative cell line HCC1937 had about 50% of the ESR1 expression as T47D. The other triple negative cell line, MDA MB 231, had very little expression and interestingly the “normal” epithelial cell line, 76N tert, also has very low ESR1 expression ($p<0.05$).

ESR2 expression in the ZR 75-1 and the 76N tert cell line is similar to the T47D cell line (Figure 2.5b). The triple negative cell lines MDA MB 231 and HCC 1937 have significantly greater ESR2 expression, with levels that are ~9-fold and ~12.5-fold, respectively, greater than T47D ($p<0.01$). The parental MCF-7 cell line has considerably lower ESR2 expression than T47D , but the decrease is not significant. The MCF-7 ERβ repressed cell line has six-fold greater ESR2 expression when compared to T47D ($p<0.01$). This is probably due to leaky expression of the ESR2 gene, even in the presence of doxycycline. Predictably, in the absence of doxycycline, the MCF-7 ERβ expressed cell line had greater than 200-fold increase in ESR2 expression compared to T47D.

Validation of Estrogen receptor beta antibodies

There was a need to evaluate several ERβ antibodies for western blotting. The primary antibodies GTX70174, PA1-311 and PA1-310B were evaluated. ERβ has a calculated molecular weight of about 55-59 kDa in western blots. The antibody from GeneTex, GTX70174, failed to show a band on a western blot. In contrast, a band was visualized with the antibody PA1-311 from Thermo Scientific, but the suspicion is that the band was non-specific binding of the antibody. Comparison of the band to the Dual Color Precision Plus Protein™ Standard (BioRad; Hercules, California) showed a product with a molecular weight of 66-69 kDa (Figure 2.6 a). Additionally, product was detected with the PA1-311 antibody in the estrogen receptor negative HeLa cells. The PA1-310B antibody detected a ~47kDa product (Figure 2.6 b) in MCF-7 cells, MCF-7 cells

transfected with ERβ1 and the T47D cells, but did not detect any product in either the ovaries from βERKO mice or in HeLa cells, which are negative for both estrogen receptors. Therefore, we used this PA1-310B antibody to quantify the ERβ protein expression in the breast cancer cell lines.

Relative intensity of Estrogen Receptors Protein

The expression of ERα (MC-20 antibody) and ERβ using the validated ERβ antibody PA1-310B were determined for all of the cell lines (Figure 2.7 & 2.8). The relative intensities of ERα (2.8 a), ERβ (2.8 b) and β-actin (2.8 c) were made relative to T47D. Protein expression was not normalized to β-actin, instead we relied on accurate quantification via Bradford assay and loading of total protein because each cell line could have different composition of cytoskeletal elements. Therefore, normalizing protein to β-actin may not reflect true expression. Each cell line is quite different in behavior and phenotype, but regardless there are no differences in β-actin (Figure 2.7 & 2.8 c).

Expression of ERα protein was the greatest in the T47D cell line and was reduced by about 50% in the MCF-7 parental cells (Figure 2.8 a). The MTO ERβ off and on cells had about half to two-thirds less ERα detected than the MCF-7 parental line, although this difference relative to the parental cell line was not significant (0.1 and 0.058). The other ERα positive cell line, ZR75-1 and the "normal-like) 76N tert, expressed 3% and 7% of T47D level of ERα. The triple negative cell lines MDA MB 231 (2%), HCC1937 (6%) and HeLa (2%) had marginal expression of ERα.

Quantification of the ERβ western blots showed that T47D and MCF-7 had the highest peak intensity (Figure 2.8 b). MTO ERβ off and on had nearly equal ERβ protein detected, ~70% of MCF-7 parental cells. The triple negative cell lines MDA MB 231 and HCC1937 had about half as much ERβ expression as T47D. The cell lines ZR75-1, 76N tert and HeLa failed to produce a detectible band on the western blot.

Transcript ratios do not directly correlate to protein ratios or levels in breast cancer cell lines

Because ERβ is proposed to modulate the proliferation activity of ERα (Li et al., 2004) and relative abundance of the receptor vary in the cell lines, we wanted to be able to determine ratios of receptor expression. Our objective is to classify the cell lines based on receptor ratios as those that express both receptors, or are predominantly ERα-expressing or ERβ-expressing to correlate to the estrogen response. Therefore, expression of ESR2: ESR1 transcript, as well as ERβ: ERα protein in each cell line was determined. Presuming that ESR2: ESR1 and ERβ: ERα were 1:1 in T47D cells, although this is not likely, we can determine relative ratios of estrogen receptor transcript and protein in each of the other cell lines (Figure 2.9). Although transcript of ESR1 is equal between T47D, MCF-7 and MTO cells (Figure 2.5) the expression of ERα is not (Figure 2.8 a) ESR2 transcript was significantly higher in MDA MB 231, MTO and HCC1937 cells, but surprisingly, ERβ protein was not increased. These differences are reflected in the different ratios of expression between transcript

and protein in each cell line. For instance, in MCF-7 cells, the ratio of ESR2: ESR1 is 0.11, meaning for each unit of ESR2 (relative to T47D) there are 10 units of ESR1 (relative to T47D). However, the MCF-7 ERβ:ERα ratio shows 2.77 relative units of ERβ to each relative unit of ERα. The MTO ERβ repressed cell line ratio shows that there are about 5 units of ESR2 for every 1 unit of ESR1 and a little over 3 units of ERβ:ERα relative to T47D. ESR2 transcript in the MTO ERβ expressed cell line had over 200 relative units of ESR2: ESR1, yet surprisingly, peaked at only 5 units of ERβ to ERα protein relative to T47D. The triple negative cell lines MDA 231 and HCC1937 expressed 182.79 and 23.64 units respectively of ESR2 to ESR1 relative to T47D. ERβ protein levels were high compared to T47D at 15. 47 and 7.68 units of ERβ ratio, relative to T47D. Based on these relative ratios of ESR2: ESR1 and ERβ: ERα, we have classified the cell lines as those that express both receptors (T47D, MCF7, MTO repressed); those that express a higher ratio of ERβ: ERα (MTO expressed, MDA 231, HCC1937); and those that have poor expression of ERs (ZR75-1 and 76N tert).

Effect of ICI on ERα in T47D cells

In an attempt to modulate ERα expression we used both ICI 182,780 and E2 treatment. ICI is known to decrease ERα protein (Oliveira et al., 2003) and may stabilize ERβ (Montanaro et al., 2005). ICI and E2 were expected to decrease ERα expression. ICI binds to ERα to target it for degradation. E2 should trigger a negative feedback loop, reducing the amount of ERα protein

expression. We found that neither ICI nor E2 decreased ERα protein measured in T47D cells on a western blot (Figure 2.10 a). Quantification of ERβ did demonstrate stabilization of ERβ protein by ICI treatment, but not with E2 or ICI + E2 treatment (Figure 2.10 c). Quantification of ERα and β-actin showed no significant difference between T47D controls, ICI, E2, or ICI + E2 treatments (Figure 2.10 b & d).

Discussion

It is obvious that mRNA expression and protein expression do no correlate with each other. We found that ESR1 expression is equivalent between T47D, MCF-7 parental and MTO cells. But ERα protein in the MCF-7 parental line was only 50% of the amount of ERα protein relative to T47D. MTO cells were found to have only about 20-25% of ERα relative to T47D. ESR2 expression is significantly increased by >6 fold in MTO cells and further increased by greater than 200 fold in the MTO cells when expression is relived by removing doxycycline relative to T47D. However, ERβ protein expression is nit increased in the cell lines relative to T47D cells, even though transcript is abundant.

Our findings confirm that there is no clear correlation found between Estrogen Receptor mRNA and protein expression in breast cancer cell lines. This result corroborates with published data in which mRNA expression in thousands of genes did not predict corresponding protein levels in MCF-7, MDA MB 231 and three other breast cancer cell lines (Cifani, Kirik, Waldemarson, & James, 2015). This outcome is partially attributed to the dynamic mRNA and protein synthesis

environment in which quantitative RNA levels may be fundamentally detached from protein half-life in the cellular environment (Schwanhausser et al., 2011).

The published data agrees with our results of positive ERα status in MCF-7 and T47D cells (Kao et al., 2009; Tong et al., 2002). But the literature differs with regard to ERβ expression in MCF-7 and T47D cells (Bulzomi et al., 2012; Davies et al., 2004). Our results suggest that both cell lines express ERβ. Further, there were two interesting results in regard to the characterization of the cell lines. One was that our results show that the ERα positive cell line ZR75-1 expressed neither ERα nor ERβ. The second is, that although the MTO ERβ cell line with ERβ expressed greatly increased transcript, the protein was not increased. This is in contrast to work done in other labs where the MTO ERβ off showed no ERβ protein expression and the MTO ERβ on had a clearly defined band for ERβ expression using a cocktail of 1:1 of the ERβ antibodies 14C8 and 7B10.7 both from GeneTex (Hodges-Gallagher, Valentine, El Bader, & Kushner, 2008). Another surprising result was that neither ICI nor E2 decreased the protein expression of ERα, but ICI did appear to stabilize ERβ.

Some of the controversy in the literature comes from the detection of ERβ in western blots. We tried three antibodies: GTX70174 did not have any detectable bands, PA1-311 detected an antigen with a higher than expected molecular weight. PA1-311 antibody also detected antigen in the HeLa cell line, which are known to be ERβ negative. PA1-310B detected an antigen with a lower molecular weight than expected. PA1-310B was used throughout this study, but

its specificity for the ERβ receptor is also questionable due to the lack of a strong band in the MTO cells with ERβ overexpression. Another curiosity is that PA1-310B indicated strong expression of ERβ protein in the MCF-7 cell line, although the ESR2 transcript in this cell line is extremely low. Therefore our confidence that this antibody is detecting ERβ is also low.

It is standard in clinical practice to define treatments for breast cancer based on their hormone receptor status. About 15% of breast cancer is classified as triple negative, so treatments that rely on hormone receptors are not an option for these patients. Also, receptor testing does not take in to account the ER subtypes. ERβ is thought to be a tumor suppressor as well as modulate the transcriptional effects of ERα. ERβ has been found in triple negative breast cancer. In this study we attempted to confirm the presence and the balance of the ERα and ERβ in our reference cell lines. This work shows that two TNBC cell lines do indeed have ESR2 expression on the mRNA and ERβ protein levels. The TNBC lines MDA MB 231 and HCC1937 expressed 182-fold and 24-fold more ESR2 than the ESR1 positive cell line T47D (Figure 2.10). These cell lines also expressed relatively high ratios (~15 and 8 fold more) of ERβ protein in western blots (Figure 2.10) than T47D. Therefore, targeting ERβ may have a clinical benefit for TNBC.

Estrogen Receptor α	T47D	MCF7	ZR75-1	MDA MB 231	HCC 1937
Tong et.al., 2002	+	+	+	-	
Kao et.al., 2009	+	+	+	-	-
Subik et.al., 2010		+	+	-	
Ford et.al., 2011	+	+	+	+	
DL Holliday et.al.,2011	+	+	+	+	
Estrogen Receptor β	T47D	MCF7	ZR75-1	MDA MB 231	HCC 1937
Treeck et al., 2008		+		+	
Poola et al., 2002		+		+	
Davies et al., 2004	+	+		+	
Bulzomi et al., 2012	-	-			
Hodges-Gallagher et. al., 2007		-			

Figure 2.1 Estrogen Receptor alpha (a) and beta (b) status charts from several sources demonstrate conflicts. T47D, MCF-7 cells test positive for both ERα and ERβ while MDA MB 231 and HCC1937 cells could be both ERα positive or negative depending on the source.

ESR1 (ERα)	left- primer 5'-AGCCTACGAGCACCTGA-3'
	right- primer 5'-GGTTTGGCTGGGGTAACTG-3'
ESR2 (ERβ)	left-primer 5'-TGGAGTCTGGTCGTGTGAAG-3'
	right-primer 5'GTCGGCACTTCTCTGTCTCC-3'

Figure 2.2 Primer sequences used in qPCR.

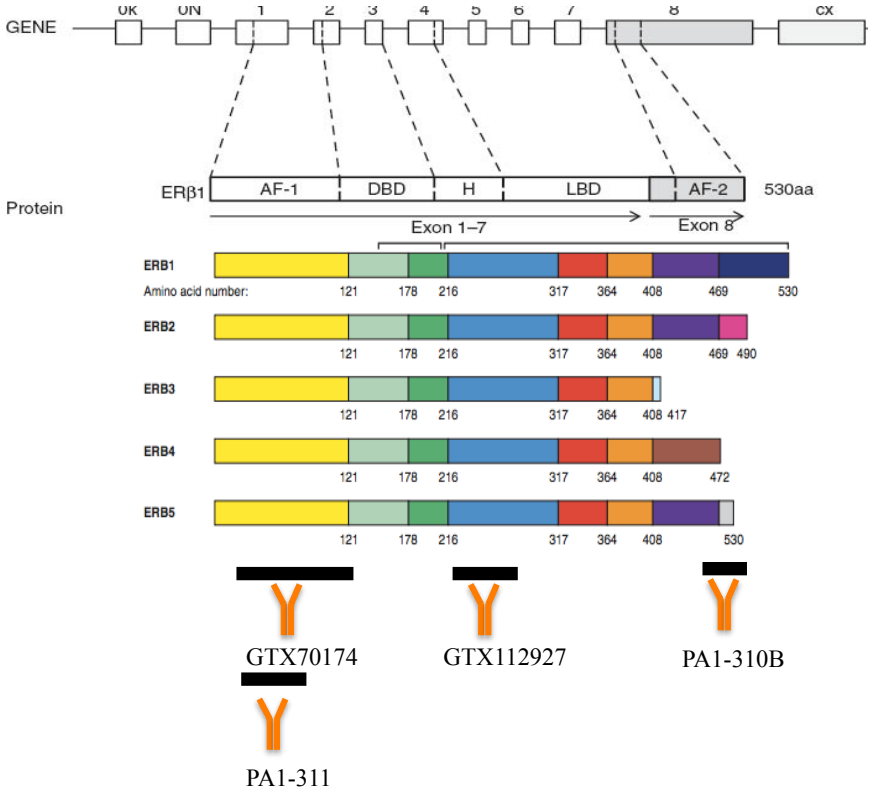


Figure 2.3 Diagram of Estrogen Receptor beta isoforms with corresponding antibody location on the receptor. ERβ has five mRNA splice variants. ERβ-cx, cannot bind ligand, due to a change in helix 12 (LBD), resulting from the alternative splicing of exon 8 (Ogawa et al., 1998).

Adapted from Adam W. Nelson, Wayne D. Tilley, David E. Neal, and Jason Carroll “Estrogen receptor beta in prostate cancer: friend or foe?” Endocr Relat Cancer ERC-13-0508, doi:10.1530/ ERC-13-0508 first published on 8 January 2014.

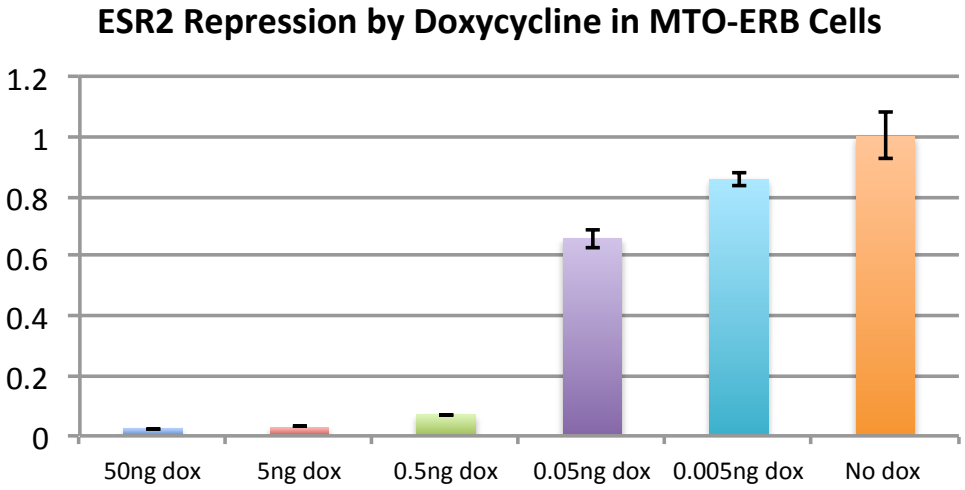
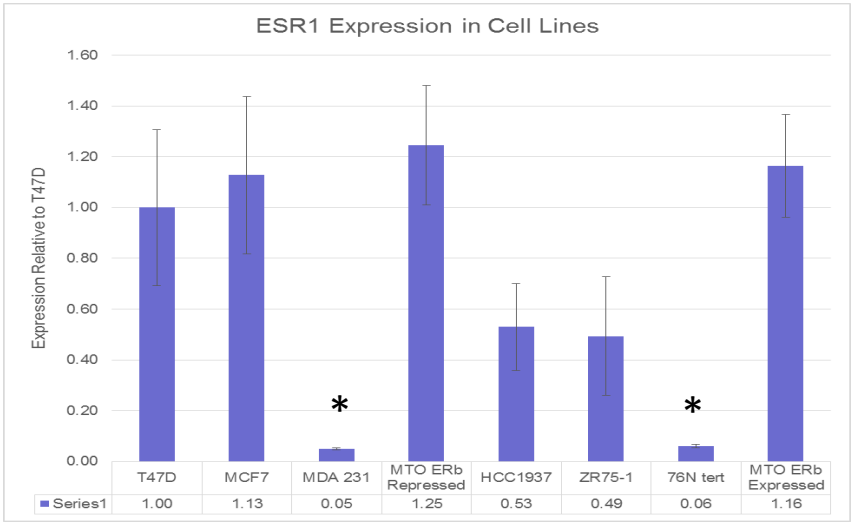


Figure 2.4 ESR2 Repression by Doxycycline in MTO-ERβ Cells. The cell line MTO-ERβ (MCF7 tet off ERβ) has a repressible Estrogen Receptor beta. In the presence of 50ng doxycycline (dox), expression from a tet-inducible promoter is reduced. As the concentration of dox is reduced to 0.005ng, the expression of ERβ increases.

a



b

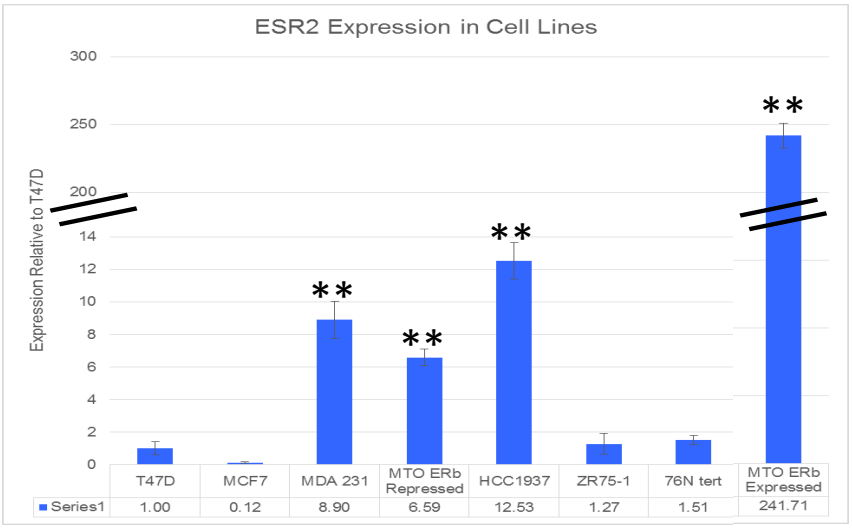


Figure 2.5 ESR1 (a) and ESR2 (b) Expression in Human Breast Cancer Cell Lines. Expression of the receptors is relative to T47D.
* $p<0.05$; ** $p<0.01$

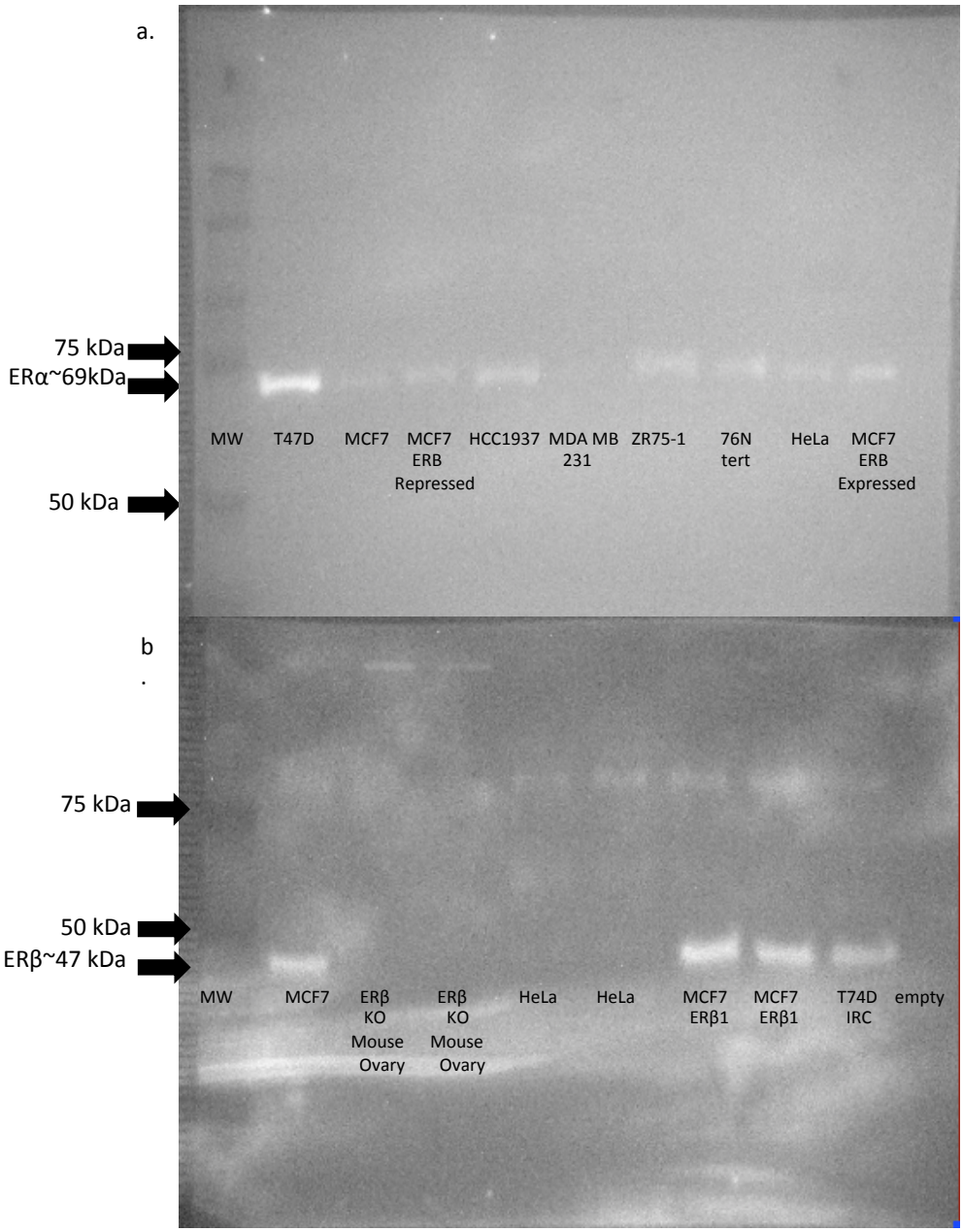


Figure 2.6 Western Blot comparing PA1-311 and PA1-310B ERβ antibodies. 20µg of whole cell lysate loaded per well. Lane 1 on both blots is the molecular weight marker. (a) PA1-311 ERβ antibody detected a band at ~69kDa. (b) PA1-310B ERβ antibody detected a band at ~47 kDa.

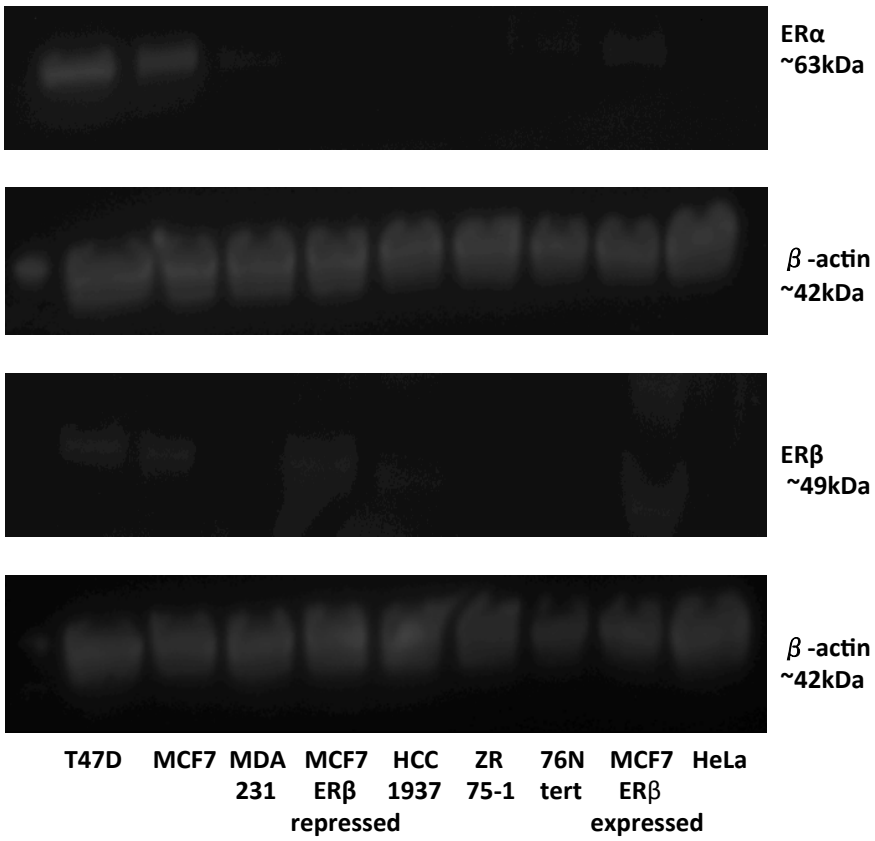
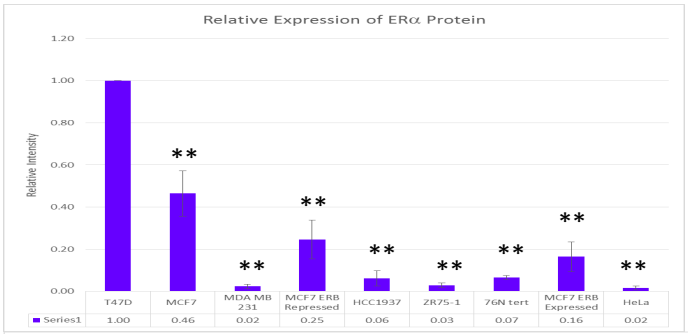
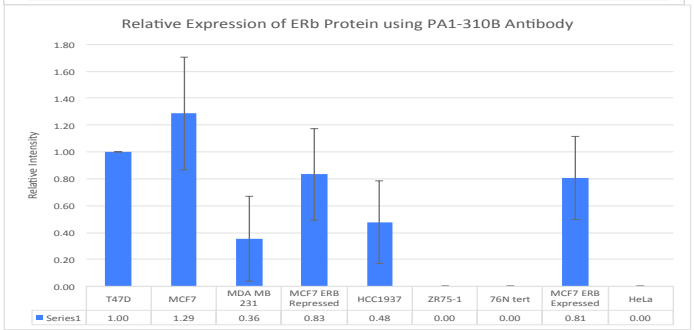


Figure 2.7 Representative western blot for ERα, ERβ and β-actin. Total proteins were resolved using SDS-PAGE and antibodies for ERα (MC-20), ERβ (PA1-310B) and β-actin loading control.

a



b



c

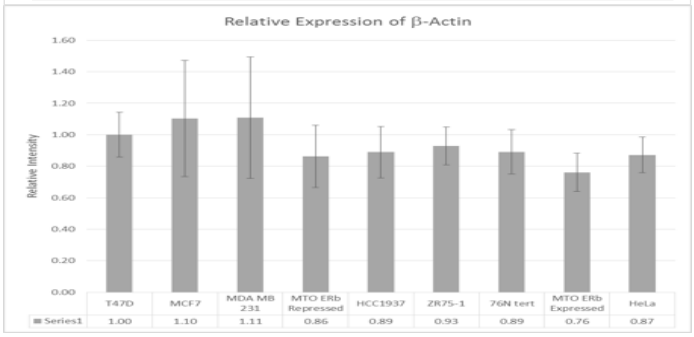


Figure 2.8 Quantification of ERα, ERβ and β-actin in Human Breast Cancer Cell Lines. Representing four (ERα and β-actin) and two (ERβ) separate experiments run independently using different samples of the representative cell lines. ** $p<0.01$

Express both Estrogen Receptors		
	ESR2/ESR1	**ERβ/ERα**
T47D	1.0	1.02
MCF7	0.11	2.77
MTO ERβ repressed	5.29	3.4
Express high ratio of ERβ: ERα		
MTO ERβ expressed	207.61	4.91
MDA231	182.79	15.47
HCC1937	23.64	7.68
Express negligible ERs		
ZR75-1	2.59	0.00
76N tert	25.36	0

Figure 2.9 Relative Ratios of ESR1: ESR2 and ERα: ERβ in Human Breast Cancer Cell Lines.

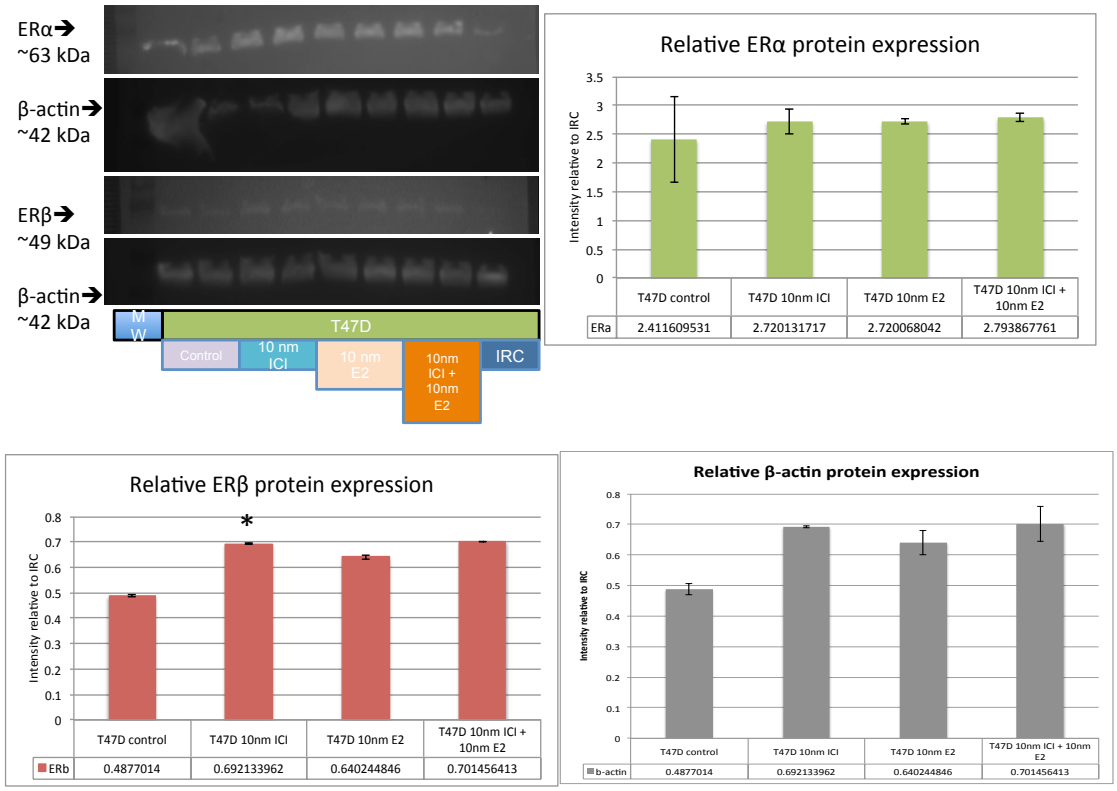


Figure 2.10 Western blot and Quantification of the relative expression of ERα, ERβ and β-actin proteins in T47D cells treated with Estrogen Receptor agonists (a). Quantification of the western blot measuring the effect of ICI on ERα (b), ERβ (c) and β-actin (d) in T47D cells. *p< 0.05

CHAPTER 3

COMPARISON OF RESPONSES TO DIFFERENT ESTROGEN RECEPTOR AGONISTS IN BREAST CANCER CELL LINES *IN VITRO*.

Introduction/Rationale

Only 15-30% of luminal epithelial cells express estrogen receptors. In response to estrogens, these cells generate local factors to regulate cell fate and the development of neighboring cells. ER mediated transcription of genes is regulated by ligand binding of a specific estrogen receptor agonist (Leitman et al., 2010; Paruthiyil et al., 2011). Stimulation of ERα, through the binding of its agonists PPT or E2, mediates cell proliferation (Helguero, Faulds, Gustafsson, & Haldosen, 2005; Sotoca et al., 2008). ERβ expression and agonist stimulation slows the growth of cells (Hodges-Gallagher et al., 2008; Paruthiyil et al., 2011). When the balance of the receptors in the cell leans towards more ERβ than ERα, proliferation is repressed (Gougelet, Mueller, Korach, & Renoir, 2007). I have observed that increasing the balance of ERβ expression does abate proliferation through my own experience cultivating the MTO ERβ cells without dox when compared to MTO ERβ cells maintained in dox.

Three cell lines stood out as examples of ERα and ERβ expressing, predominantly ERα expressing or predominantly ERβ expressing. The complement of Estrogen Receptors in cells is important because the ratios of receptors influences cell growth (Helguero et al., 2005) as well as the ability of a cell to respond to specific Estrogen Receptor agonists (Marzagalli, Casati,

Moretti, Montagnani Marelli, & Limonta, 2015). T47D was chosen because it expressed both ERα and ERβ in qPCR and western blots. Western blots using the cell line MCF-7 showed that it had ERβ, but mRNA expression suggests that MCF-7 is primarily an ERα expressing cell line because little ESR2 is expressed. Also, we do not have a lot of confidence in the ERβ antibody (PA1-310B), as discussed in chapter 2, therefore we regard MCF-7 as primarily ERα. Finally HCC1937 was chosen because both the ERβ protein and the ratio of ESR2: ESR1 in qPCR was surprisingly high, which led us to regard it as a predominantly ERβ expressing cell line.

The objectives of these experiments using the cell lines are to compare responses in each cell line type (ERα and ERβ expressing, ERα expressing and ERβ expressing) to different receptor agonists in terms of proliferation responses and potential genome surveillance. ERα and ERβ directs the transactivation of estrogen response genes in the cell including genes that modulate proliferation and genomic surveillance (Lattrich, Juhasz-Boess, Ortmann, & Treeck, 2008). The progesterone receptor is one of the downstream targets of ERα and its upregulation is correlated to a ligand-activated ERα (Flötotto et al., 2004; Saji et al., 2002). Amphiregulin (AREG) is an important growth factor that is also induced by estrogen through ERα stimulated signaling (Ciarloni, Mallepell, & Brisken, 2007). This member of the epidermal growth factor (EGF) family signals in a paracrine fashion through the EGF receptor to stimulate proliferation. AREG upregulation is also associated with hyperplasia in breast cancer in humans and

mice (Lee et al., 2007; Niemeyer, Spencer-Dene, Wu, & Adamson, 1999). The expression of TGFβ2 and CEBPd indicate induction of genomic surveillance. Transforming growth factor- beta 2 (TGFβ2) is a cytokine that once bound to its membrane receptor, initiates the intercellular recruitment of a complex of activated Smad proteins that interact with DNA to transactivate genes involved in cell cycle arrest and apoptosis (Massagué, 2008). CEBPd, a member of the CCAAT transcription enhancer binding proteins, has been indicated as a good prognostic marker for long progression free survival in patients with ERα positive breast cancer (Mendoza-Villanueva et al., 2016). Also, in MCF-7 and other breast cancer cell lines, CEBPd was also found to promote differentiation and inhibit growth through the down regulation of cyclins (Pawar et al., 2010). The quantification of these transactivation markers after the acute agonist treatment, with different complements of Estrogen Receptors as context, will help elucidate ER mediated cell fate.

Materials and Methods

Cell Culture: All cells were cultured at 37°C and 5% CO2. Each Cell line was expanded in their standard Dulbecco's Modified Eagle's Medium/Nutrient mixture F1-12 Ham (DMEM:F12, Sigma-Aldrich; St. Louis, MO) with 15 mM $NaHCO_3$ and 25 mM Hepes. Three days before plating for each treatment (in triplicate), the media was switched to phenol red-free (prf) DMEM:F12 media with 5% Charcoal Stripped Fetal Bovine Serum (CS-FBS, Sigma-Aldrich; St. Louis, MO). Cells were plated either at 500,000 cells/well or 1,000,000 cells/well in a six well plate (Cell

Treat; Shirley, MA) at 50-60% confluence in order to achieve optimum 75-80% cell density at time of harvest. One day after plating the serum was reduced to 1 or 2% CS-FBS. After incubating for 24 hours in 1 or 2% CS-FBS media, cells were treated with the agonists at the concentrations indicated (Figure 3.1) in prf DMEM:F12 media with 1 or 2% CSS. Cells were treated with either E2 (Sigma Aldrich; Cat# E27858), PPT (R&D Systems; Cat#1426-50), ERB041 (R&D Systems; Cat# 4276-50 in combination with ICI (Sigma Aldrich; Cat# I4409-25MG). The cells were harvested for mRNA or lysates after 24 hours of treatment. To repress the expression of ERβ in the MCF-7 tet off cells, cells were maintained in 50 ng/ml Doxycycline (Dox)(Sigma-Aldrich; St. Louis, MO).

mRNA isolation and RT-qPCR: mRNA isolation and RT-qPCR was described previously in chapter 2 and primer pairs are listed in Figure 3.2. Gene expression is shown relative to an IRC and the two-sided Student's t-test was used to determine differences between each agonist treatment together with ICI against the control treated with ICI. P-values of <0.5 (*) was considered significantly different; <0.01 (**) considered highly significantly different. Error bars indicate SEM.

Results

Treatment with 10nM ICI 182,780 suggests potential contaminating estrogens in cell culture

Although, there was a significant increase in AREG expression in T47D cells treated with Estrogen Receptor agonists E2, PPT and ERB041 compared to untreated T47D cells (Figure 3.3 a), treatment with 10nM ICI also significantly decreased AREG expression. We found that 1nM E2 treatment as well as all doses of PPT significantly increased AREG expression. ERB041 was able to increase AREG expression in T47D cells only at the lowest dose (20nM), while the higher doses were not significant. Whereas the differences are considered significant with the t-test, the fold change increase is small, with between 0.5 to 2 -fold increments and there was no observable dose dependent effect. We found no significant effect in terms of PR expression in MCF-7 cells using the same Estrogen Receptor agonists (Figure 3.3 b). However, treatment with 10nM ICI also significantly reduced PR expression relative to the untreated control. The reduction in expression of AREG and PR expression in the presence of 10nM ICI signifies that the culture conditions may contain an unknown estrogenic compound that is increasing background AREG and PR expression in the T47D and MCF-7 cells.

Effect of Agonists on gene expression in T47D cells

Because we detected background estrogenic activity that could be reduced with 10nM ICI, T47D cells were treated with ICI in conjunction with agonists to observe differences in mRNA expression. E2 treatment significantly increased the expression of AREG in a dose dependent manner (Figure 3.4 a) Although 1nM PPT significantly decreased AREG expression ($p=0.03$); PPT at the highest doses, similar to E2, increased expression of AREG in a dose dependent manner. Interestingly, the ERβ agonist, ERB041, significantly decreased the expression of AREG in T47D cells, signifying that specific activation of ERβ repressed this growth factor. We also analyzed the expression of two presumed estrogen regulated genes that could potentially restrict cell growth. TGFβ2 was significantly suppressed by E2 (Figure 3.4 b) both at 1nM and 10nM E2. The background xenoestrogens in the culture conditions also suppressed TGFβ2 expression in the control without ICI, but 10nM ICI releases this repression. Low doses of PPT did not repress TGFβ2, but high doses (500nM) of PPT significantly repress TGFβ2. Although the expression of TGFβ2 at the highest ERB041 concentrations (5000nM) was decreased, this may be mediated through ERα, rather than ERβ because high concentrations of ERB041 can have agonist effects on ERα. CEBPd, a potential marker for cell cycle inhibition also appeared to be inhibited in the control cells without ICI and again repression was relived by treatment with ICI (Figure 3.4 c). High doses of E2 (10nM) and PPT (500nM) were able to suppress CEBPd expression while lower

doses of the ERα agonists did not significantly change expression. ERB041, the ERβ agonist, increased CEBPd expression in T47D cells at 500nM, but not at the low or high concentrations.

Effect of Agonists on gene expression in MCF-7 cells

AREG expression by background estrogens is again blocked by ICI treatment in the MCF-7 cells (Figure 3.5 a). E2, at both the 1nM and 10nM concentrations, was able to significantly increase AREG expression in a dose dependent manner. Interestingly, all three doses of PPT increase the expression of AREG in the presence of ICI, but there was a slight decrease in expression, from about 20 fold at the highest dose (500nM) to about 17-fold difference (50nM) over ICI control. ERB041 at lower doses does not increase expression of AREG except at the highest dose (5000nM), however, at high dose (5000nM), ERB041 did increase AREG expression significantly, probably because it is able to cross-react with ERα. There was no significant TGFβ2 (Figure 3.5 b) or CEBPd (Figure 3.5 c) response to the agonists even though the 500nM dose of PPT seems to decrease TGFβ2 in MCF-7 cells.

Effect of Agonists on gene expression in HCC1937 cells

Next, we used the Estrogen Receptor agonists in HCC1937 cells to evaluate their transcriptional responses. There was no significant change is expression of AREG (Figure 3.6 a), TGFβ2 (Figure 3.6 b) or CEBPd (Figure 3.6 c) in response to agonists. Appraisal of the ct values in the qPCR showed that there were several factors that caused variability in our results. There were a

large number of outlying ct values as well as a wide range of ct value variance in the samples that contributed to the standard error mean. More specifically, the variance within treatment groups was mirrored for the gene expression of each of the three genes, suggesting that quantification of the RNA was inaccurate. This experiment should be repeated because we expect that HCC1937 cells, a predominantly ERβ expressing cell line, will not be able to be stimulated by the agonists and therefore will not be able to express the ERα regulated gene AREG. Whereas, the ERβ agonist ERB041 may increase TGFβ2 and CEBPd expression, E2 and PPT should not be able to repress the expression of these genes.

Comparison of AREG, TGFβ2 and CEBPd expression to E2 treatment in three cell lines

Because we chose cell lines to represent three different estrogen receptor ratios: (1) expressing both ERα and ERβ, (2) primarily ERα expressing and (3) primarily ERβ expressing, we wanted to compare estrogen responses across the three cell lines. The ERα and ERβ expressing T47D cells as well as the ERα expressing MCF7 cells responded to increasing doses of E2 by expressing AREG in a dose dependent manner (Figure 3.7 a). There was a highly significant ($p < 0.001$) difference of in magnitude of AREG expression in both the 1nM and 20nM E2 treated T47D and MCF7 cells, such that AREG induction by E2 is greatest in the MCF-7 cell line, which expresses primarily ERα. We found that the

predominantly ERβ expressing cell line HCC1937 had significantly reduced AREG expression compared to the cell lines with ERα.

Curiously, TGFβ2 was significantly increased in the ERα expressing MCF-7 cell line in comparison to both the ERα/ERβ expressing cell line T47D an the ERβ alone expressing HCC1937 cell line (Figure 3.7 b). There was no difference in TGFβ2 expression between T47D cells and HCC1937 cells. Interestingly, expression of CEBPd is significantly greater in cells that express ERβ only (HCC1937) relative to cells that also express ERα and ERβ (T47D) or ERα (MCF7) only (Figure 3.7 c).

Discussion

In our initial experiments, we found that ICI significantly decreased the expression of AREG and PR, known ERα responsive genes, suggesting that background estrogens were contributing to ERα mediated gene transcription. This background activity obscured our ability to measure dose dependent responses (AREG) or any responses to agonists at all (PR).

Finding background estrogen activity in our cell culture system is a hazard of working with plastics that may leach xenoestrogens into our cell culture dishes (Sax, 2010). These xenoestrogens mimic the activity of naturally occurring estrogen to the point of obscuring estrogenic activity in signaling pathways on the genomic and non-genomic level (Jeng, Kochukov, & Watson, 2010). This is important because cell culture supplies could be contributing to a false result. Investigators are aware of the estrogenic effects of phenol red and estrogen in

serum, but other factors could be present and should be tested for and controlled in estrogen related research models. Our results show that is important to test for background estrogen activity in cell culture, and in our case, ICI decreased significant background estrogenicity.

Using ICI to block background estrogen, we were able to detect significant dose-dependent responses to the agonists in the cell line that expressed both receptors – T47D. The proliferation maker, AREG, increased by E2 and PPT, but was decreased by the ERβ specific agonist ERB041. The expression of growth restrictive markers (TGFβ2 and CEBPd) decreased in the presence of E2 and PPT. Whereas, ERB041, the ERβ agonist may stabilize or increase expression. This indicates that cells that express both ERα and ERβ have a balance of proliferation and growth restriction through the two receptors. Further, cells that express primarily ERα, like MCF-7, also induce dose dependent increases in AREG expression in response to E2 and PPT. However, none of the agonists induced any change in expression of TGFβ2 or CEBPd.

Interestingly, the magnitude of AREG expression in the primarily ERα cell line was significantly increased relative to T47D cells which express both estrogen receptors. This indicates the importance of the presence of ERβ to modulate expression of AREG and proliferation. Although the analysis for HCC1937 needs to be repeated, the current data demonstrates two characteristics (1) AREG expression is not induced and is relatively low in the primarily ERβ HCC1937 cell line (the cells grew painfully slow) and (2) the

magnitude of CEBPd is elevated in the HCC1937 line relative to both ERα and the ERα/ERβ expressing cell lines.

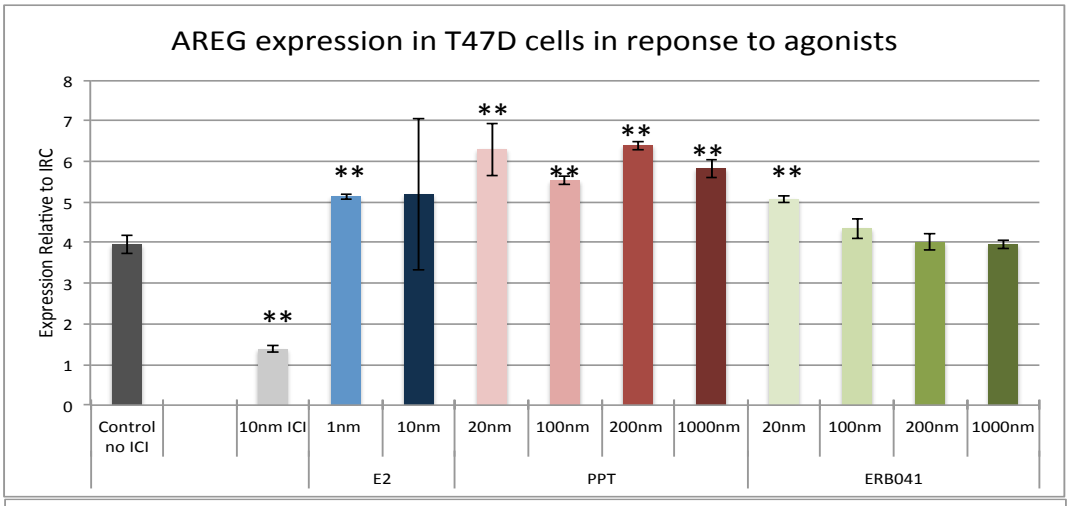
Treatment	Working Concentration	Receptor Action/Affinity
ICI 182,780	10 nM	ERα Antagonist
17 β-estradiol (E2)	1ηM or 10ηM	Equal agonist/affinity for both receptors
4,4',4''-(4-Propyl-[1*H*]-pyrazole-1,3,5-triyl)*tris*phenol (PPT)	5ηM - 1000ηM	ERα Agonist
7-Ethenyl-2-(3-fluoro-4-hydroxyphenyl)-5-benzoxazolol (ERB041)	50ηM - 5000ηM	ERβ Agonist

Figure 3.1 Agonist Table. Cell culture treatments for all experiments at the concentrations indicated.

Amphiregulin (AREG)	left-primer 5'-CGGAGAATGCAAATATATAGAGCAC-3'
	right- primer 5'-CACCGAAATATTCTTGCTGACA-3'
Progesterone Receptor (PGR)	left-primer 5'-TTTAAGAGGGCAATGGAAGG-3'
	right- primer 5'-CGGATTTTATCAACGATGCAG-3'
TGFb2	left-primer 5'-ATAGACATGCCGCCCCTTCTT-3'
	right- primer 5'-CTCCATTGCTGAGACGTCAA-3'
WNT5A	left-primer 5'-GCTTTACTCTTTCATTGTTGGGA-3'
	right- primer 5'-GGGAGACCAGAGGAAAGGAT-3'

Figure 3.2 Primer pairs used in qPCR

a



b

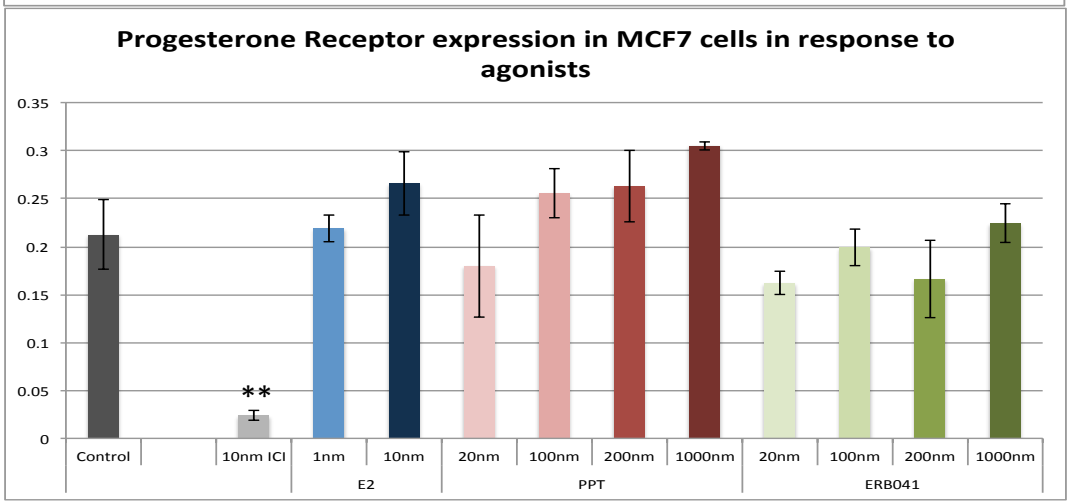


Figure 3.3 qPCR for AREG in T47D cells (a) and PR expression MCF7 cells (b) without ICI treatment. * p <0.05; ** p<0.01

a

b

c

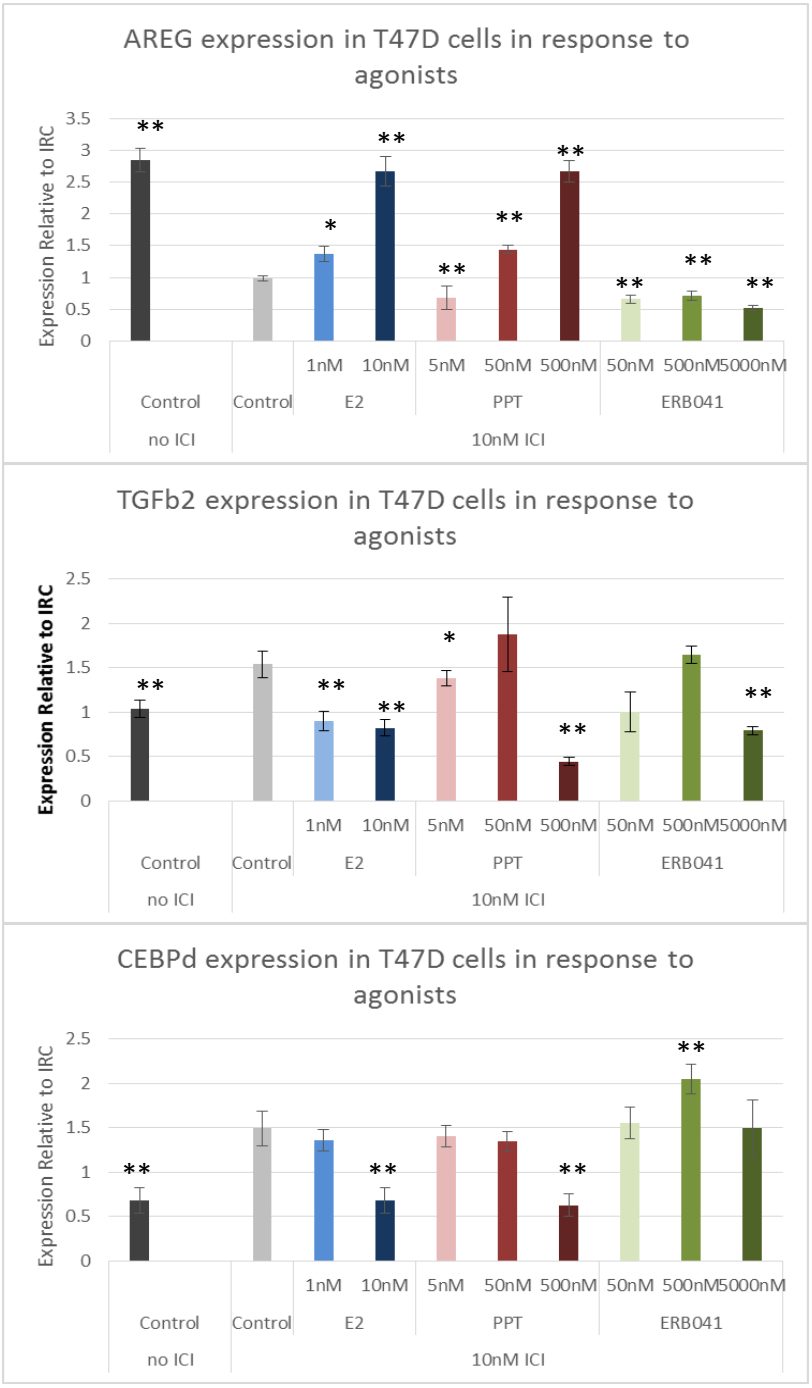
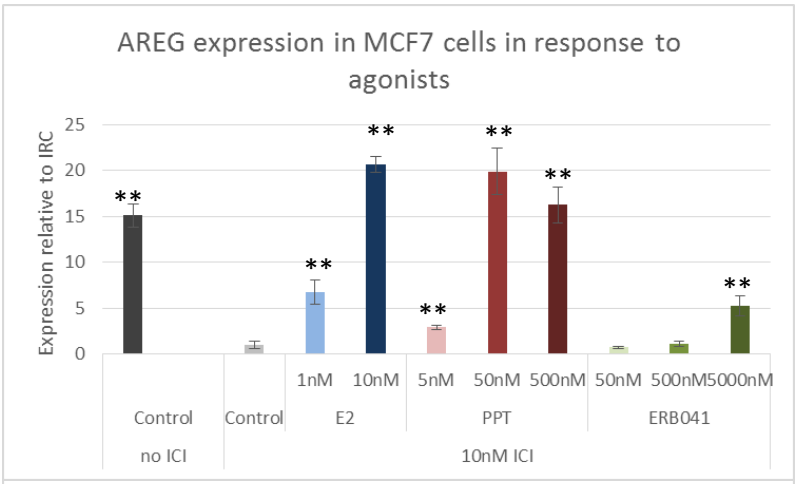
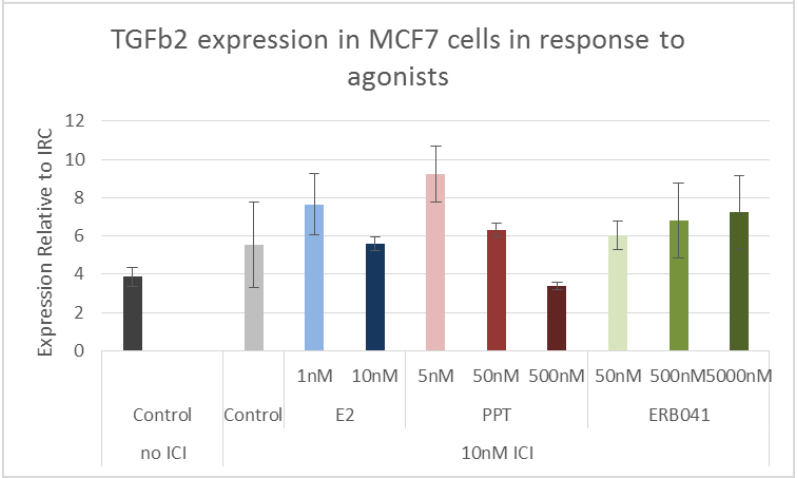


Figure 3.4 RT-qPCR in T47D cells treated with Estrogen Receptor agonists to measure AREG (a), TGFβ2 (b) and CEBPD (c) expression. * p< 0.05; **p< 0.01

a



b



c

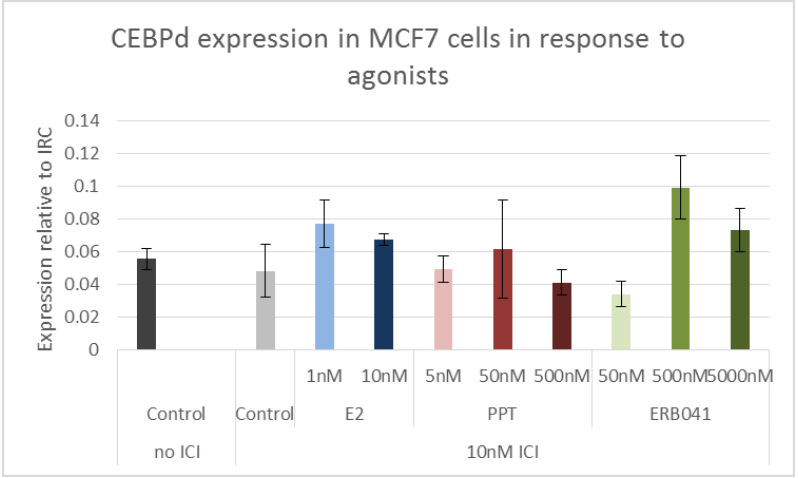
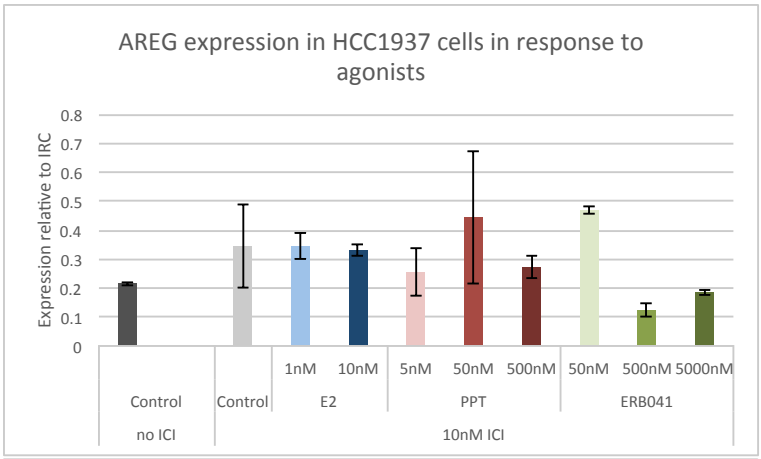
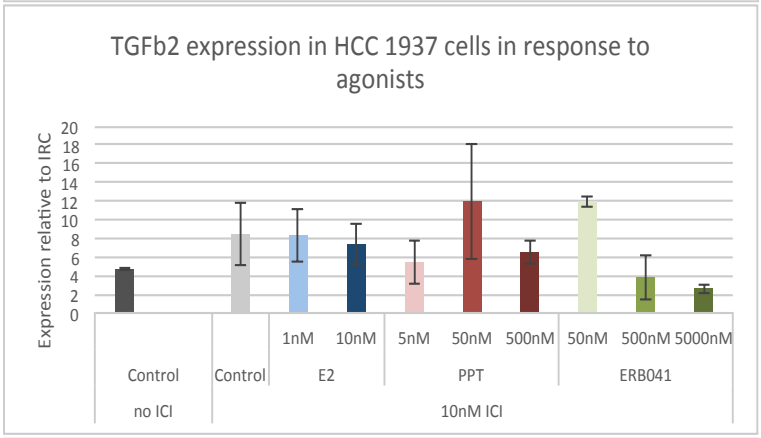


Figure 3.5 RT-qPCR in MCF7 cells treated with Estrogen Receptor agonists to measure AREG (a), TGFβ2 (b) and CEBPD (c) expression. * p< 0.05; **p< 0.01

a



b



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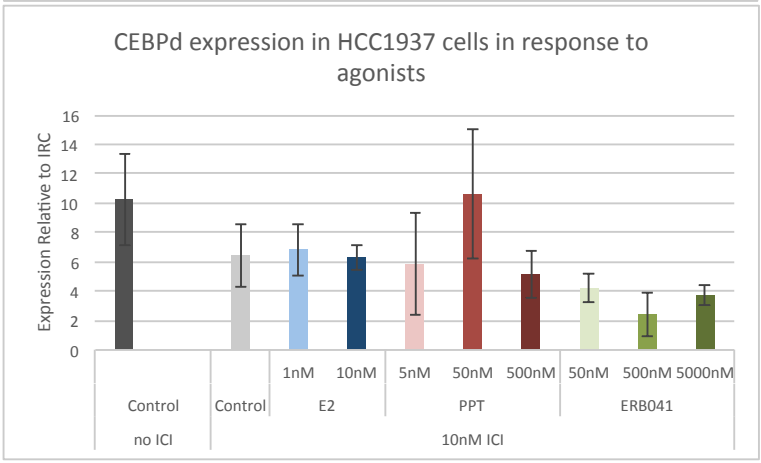

Figure 3.6 RT-qPCR in HCC1937 cells treated with Estrogen Receptor agonists to measure AREG (a), TGFβ2 (b) and CEBPD (c) expression.

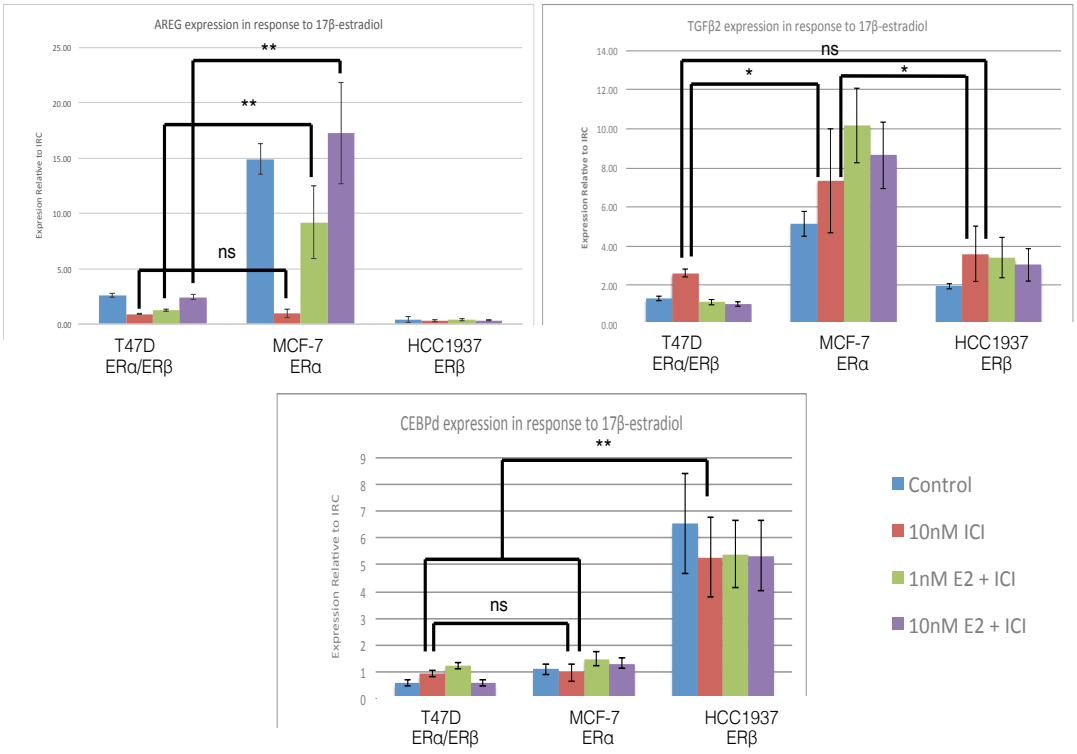

Figure 3.7 Comparison of RT-qPCR expression in three cell lines
* p< 0.05; **p< 0.01

CHAPTER 4

THE βERKO MOUSE MODEL TO EXPLICATE THE HISTOLOGIC AND GENE EXPRESSION PROFILES OF ACUTE AGONIST EXPOSURE IN MOUSE MAMMARY TISSUE FOR PROLIFERATION AND MARKERS OF SURVEILLANCE.

Introduction/Rationale

Humans and mice have a 95% exon homology and we share many of the same genetic diseases (Batzoglou, Pachter, Mesirov, Berger, & Lander, 2000). Genetic variation in humans and mice can contribute to the development of, or resistance to tumors. Mammary tumor susceptibility is different among women and mouse strains. Some women have an alteration in their tumor suppressor proteins, like BRCA1, that allows unrepaired DNA damage that leads to tumor formation. This is mirrored in some mouse strains, like BALB/c, which are more genetically susceptible to mammary tumorigenesis (Kuperwasser et al., 2000). In contrast, the C57BL/6 are resistant to mammary tumors, even in mammary tumor induction models including Mouse Mammary Tumor Virus Infection (Okeoma, Petersen, & Ross, 2009), gamma-irradiation, and even when the tumor suppressor protein p53 is knocked out (Yan et. al., 2010).

Women vary in their estrogen receptor ratios and in their responses to hormones (Dunphy unpublished). Further, the ratios of estrogen receptors shift and the expression of ERα increases while ERβ decreases as a women age,

potentially leading to a shift in the range of ER-hormone mediated responses. Mouse strains also exhibit different ER ratios and hormone responses. For example, ERα expression is greater in BALB/c when compared to C57BL/6, while ERβ expression is similar. This means that the BALB/c mouse strain has a more unfavorable ER ratio, which may potentiate tumorigenesis through ERα mediated proliferation (Montero Girard et al., 2007). C57BL/6 mice have reduced hormone-induction of RANKL (cytokine of survival, proliferation) and ID2 (negatively regulates cell differentiation), but elevated p21 (growth arrest) relative to BALB/c mice in which p21 is decreased by hormone treatment (Aupperlee et al., 2009).

These experiments are to compare the different responses to various estrogen receptor agonists in terms of growth and radiation-induced apoptosis in the C57BL/6 mouse mammary gland in the context of different estrogen receptor ratios: Wt C57BL/6 with equal ERα: ERβ expression vs. βERKO C57BL/6 with ERα expression but no ERβ, and to compare these responses to the BALB/c strain which has high ERα: ERβ ratios. The βERKO strain of mice were developed by Krege et. al. and have a deleted exon 3, the DNA binding domain of ERβ. This deletion results in a non-functional ERβ. The experiments in the βERKOs will demonstrate the specificity of the ERβ agonists.

Materials and Methods

Animal Models: Mice colonies were maintained in standard cages with ad libitum access to water and mouse diet 5015 in an animal facility on a daily 12-hour

light/dark cycle. ERβ knockout (βERKO) mice were generated by the Korach laboratory (Krege et al., 1998) with genetic disruption in Esr2 (B6.129-*Esr2*$^{tm1.1Ksk}$) were used in our experiments. These mice were generously donated by Dr. Ken Korach. The knockout allele was maintained on a C57BL/6 background. Female mice were ear notched for the dual purpose of identification and genotyping at the time of weaning, aged 21 days. Ear notch tissue was held at -20°C until DNA extraction.

Preparation of agonist pellets: Pellets were made using silastic tubing (Fisher, # 11-189-15H) sealed with silicone (DAP Inc, # 070798006881) approximately 1.2 cm long. These were packed with either cellulose alone as a control or cellulose plus one of four compounds: control (Fischer, # AC382312500), E2 (50µg; Sigma Aldrich;Cat# E27858), PPT (400µg; R&D Systems, Cat# 1426-50), ERB041 (400µg; R&D Systems; Cat# 4276-50). The proper ratio of cellulose to hormone was combined prior to packing the capsules and then each capsule was packed with compound so each would contain the appropriate concentration of the specified agonist. The pellets were sterilized with 5kGy gamma irradiation and primed in standard phenol red-free DMEM:F12 media (Sigma Aldrich; # D2906) for 24 hours prior to implantation.

βERKO C57BL/6 female mice: Virgin female mice were ovariectomized at 8-12 weeks of age and endogenous hormones were allowed to clear for two weeks (Figure 4.1). The βERKO mice were treated with individual silastic capsules packed with 50 µg of E2, 400 µg of PPT, 400 µg of ERB041 or a control silastic

capsule containing cellulose. The silastic capsules were implanted subcutaneously into the mouse dorsum and the mice were allowed to be in contact with treatments for 96 hrs. Following the treatment period, the animals were subjected to 5 Gy whole-body ionizing radiation using a 137Cs source. Mice were given an intraperitoneal injection 4 hours post irradiation of 100-200 μl of a BrdU (Bromodeoxyuridine) -containing solution (10 mg/ml solution of BrdU in sterile 1X PBS). Tissues were harvested 6h post-irradiation. At the end of the treatment period the 4th inguinal mammary glands were harvested, the lymph nodes removed and the tissues was flash frozen in liquid nitrogen for RNA. Blood was collected and allowed to coagulate for serum. The contralateral gland was fixed for whole mounts. The third mammary gland and other tissues including skin, colon and uteri were collected and were formalin-fixed and paraffin embedded for histology. All animal procedures were in accordance with institutional and national guidelines for the use of animals and were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts-Amherst.

PCR genotyping primers design and amplicon analysis: DNA from mouse ear notch tissue was isolated using KAPA Express Extract enzyme (KAPA Biosystems; Boston, MA). In brief, tissue digestion was achieved using ~1 mm mouse tissue, PCR grade H_2O, 1U/µl KAPA Express Extract Enzyme and 10X KAPA Express Extract Buffer (KAPA Biosystems; Boston, MA) using the following parameters: 20 minutes at 75°C, 8 minutes at 95°C. Following brief

voretxing, ear notch debris was pelleted. PCR was performed using primer pairs listed in Figure 4.2. The typical 11 µL PCR reaction mix contains nuclease free water, 1X KAPA2G Fast Genotyping Mix (KAPA Biosystems; Boston, MA) , 0.5 µM mutant reverse primer, 0.5 µM wild type reverse primer, 0.5 common primer and genomic DNA template (<1 µg). The standard PCR condition was as follows: 95°C for 5 min; 95°C for 15 s, 64°C for 15 s, 72°C for 20 s for 35 cycles; 72°C for 8 min followed with denaturation for 5 minutes at 95°C. PCR products were removed from the thermocycler and maintained at room temperature for at least 5 minutes allowing for annealing. PCR products were resolved with ethidium bromide-stained 1% agarose gel.

Wholemount Preparation: Whole inguinal mammary glands (gland # 4) were spread on glass slides, fixed in Carnoy's solution (60% absolute alcohol, 30% chloroform and 10% acetic acid), stained overnight in Carmine alum solution (2g/L carmine; 10mM aluminum potassium). Glands were dehydrated in graded ethanol, cleared in xylenes and mounted on slides with permount.

Results

Previous work done in the lab by Erick Roman-Perez compared proliferation and radiation-induced apoptosis in C57BL/6 WT and βERKO mice that were treated with vehicle control or E2 (50mg) for 4 days. The WT C57BL/6, which has both estrogen receptors, had a better apoptotic response than the βERKO, which only has ERα. I will build upon the previous work in the C57BL/6

and βERKO mice by including the ER agonists PPT, ERB041 along with E2 and a control.

βERKO mouse genotyping

We developed a βERKO mouse specific genotyping protocol using one primer set consisting of a mutant reverse, wild type reverse and common primer for amplification of mouse ERβ (Figure 4.2). Our primer set was designed to produce an ERβ specific PCR product of two DNA fragments with a large size difference (~730bp and 407bp). In our experiments we found that the PCR resulted in three distinct bands for the heterozygote mice at about 700bp, 600bp and 400bp (Figure 4.3). The weaker upper band (~700bp) with heterozygote mice is due to the competition of both templates for the same primer pair. Wild type mice DNA produced a PCR product at ~600bp and ~400bp. This is in contrast to the ERβ null mice which produced two bands, one at ~700bp and the other ~600bp. There was a clear distinction between the bands however, and the genotyping results were unambiguous.

βERKO mice database

In order to track the treatment history of each βERKO mouse test subject we developed a cloud-based database (Figure 4.4). Each female mouse received an identifying number via ear notch upon weaning. This number was associated with the mouse date of birth, ovariectomy date and hormone treatment. A second number was assigned to the mice upon tissue collection. This id was used for

identification of histological samples (mammary gland whole mount, H&E and serum), assays (TUNEL and BrdU) as well as RNA samples used for qPCR.

Areg is upregulated by E2

Preliminary data from fourth gland mammary tissues in ERβ knockout (βERKO) mice show that E2 was able to upregulate the expression of Areg, a marker of ERα activated proliferation, about 150-fold over untreated control tissues (Figure 4.5). PPT, the ERα selective agonist, failed to increase Areg expression in the βERKO mouse mammary tissues. This result is in contrast to previous studies in which PPT increased Areg expression to 50% of E2. Finally, because Areg is a marker of ERα activation, we do not expect the ERβ agonist to work in the βERKO mice for two reasons: (1) βERKO mice do not have a functional ERβ receptor and (2) the ERβ agonist ERB041 cannot act through a non-functional receptor. In agreement with Areg expression, the mammary epithelial ducts are thinner for the control, PPT- or ERB041-reated relative to the E2-treated in representative whole mount images (Figure 4.6).

Discussion

It was expected that E2 would be able to activate ERα in βERKO mice. The lack of Areg response to PPT in the mouse tissues was an unexpected result. Previous work by Erick Roman-Perez measured Areg in BALB/c mice that were treated with vehicle control, E2, PPT and DPN for 4 days. E2 increased the expression of Areg almost 200-fold and PPT increased expression almost 150-fold over control, while DPN-treatment did not. In that study the Jerry lab used

sesame seed oil as a carrier for hormone injections and the hormone was prepared fresh daily from stock held at -20°C in ethanol. Because daily hormone injections with sesame seed oil could be inconsistent and required more animal handling, the Lab adapted their hormone delivery methods and began using hormone mixed with cellulose packed in silastic capsules. Since then, changes to OLAW regulations for animals were implemented that required all surgically implanted hormone delivery devices to be sterilized. That meant that PPT, which should be stored at -20deg, would be packed into silastic capsules with cellulose and irradiated (5 Gy) for 48 hours at room temperature in a Cs-137 irradiator. Following sterilization the pellets are placed into priming media overnight at 37^{o}C prior to implantation into the mice. It is possible that the extended amount of time outside of ideal storage conditions as well as the potential disruption of the chemical structure through irradiation may have led to the degradation of PPT. The experiments using PPT will need to be repeated after determining methods that retain activity.

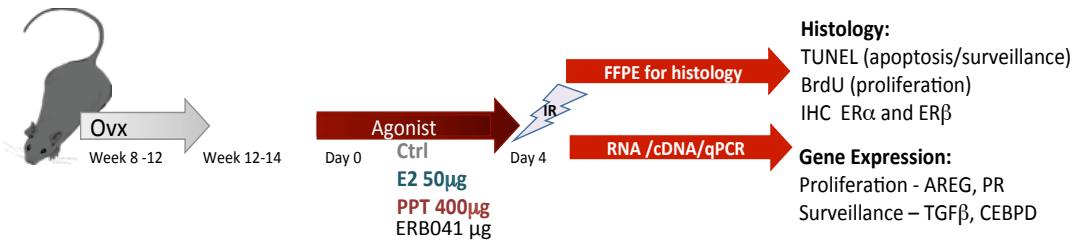

Figure 4.1 Model for βERKO mouse experiments

cDNA	Primer	Size of Product
Mutant Reverse Mouse ERβ	5'-GTTGGCAGGGAAAGTTGAAAAC-3'	730 bp
Wild Type Reverse Mouse ERβ	5'-AGTAACAGGGCTGGCACAAC-3'	407 bp
Common Mouse ERβ	5'-TCCCCAAAAGAAACATGTCC-3'	

Figure 4.2 Genotyping primers: oligonucleotide primer names, sequence, and orientation are shown.

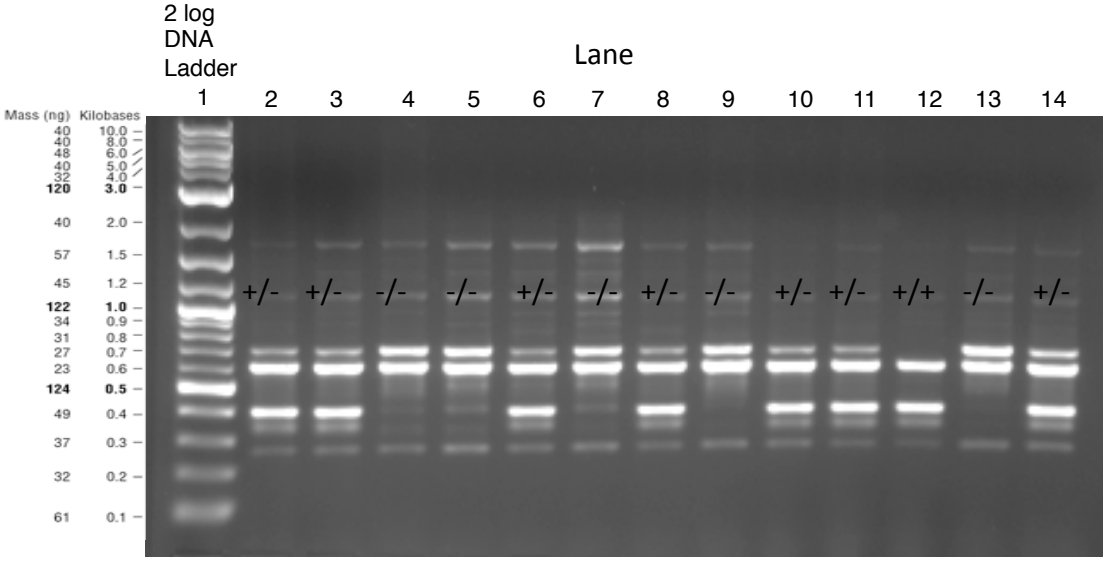

Figure 4.3 Example of genotyping results

+/+ Wild type ERβ
-/- Null (no ERβ)
+/- Heterozygous ERβ

tissue id	weight	mouse #	DOB	ovx date	treatment	date of tx	tissues collected	whole mount	H&E	AREG	TUNEL	BrdU
5115		37	11/15/15	2/11/16	no treatment	no treatment	2/25/16	x	x	6/10/16		6/17/16
5116		3	11/14/15	2/10/16	e2	3/1/16	3/4/16	x	x	6/10/16		6/17/16
5117		5	11/14/15	2/10/16	e2, lost pellet	3/1/16	3/4/16	x	x	6/10/16		
5118		11	11/14/15	2/10/16	e2	3/1/16	3/4/16	x	x	6/10/16		
5119		38	11/18/15	2/11/16	ctrl mock pellet	3/1/16	3/4/16	x	x	6/10/16		
5120		26	11/15/15	2/10/16	e2	3/1/16	3/4/16	x	x	6/10/16		
5121		39	11/18/15	2/11/16	ctrl mock pellet	3/1/16	3/4/16	x	x	6/10/16		
5123		44	11/18/15	2/25/16	ppt	3/16/16	3/18/16	x	x	6/10/16		
5124		45	11/18/15	2/25/16	ppt	3/16/16	3/18/16	x	x	6/10/16		
5125		52	12/8/15	2/25/16	ppt	3/16/16	3/18/16	x	x	6/10/16		
5126		71	12/13/15	2/25/16	ppt	3/16/16	3/18/16	x	x	6/10/16		
5127		92	12/23/15	2/25/16	ppt	3/16/16	3/18/16	x	x	6/10/16		
5145		109	1/8/16	4/7/16	no pellet ctrl	no treatment	5/6/16	x	x	6/10/16		
5146		107	1/8/16	4/7/16	no pellet ctrl	no treatment	5/6/16	x	x	6/10/16		
5147		111	1/8/16	4/6/16	no pellet ctrl	no treatment	5/6/16	x	x	6/10/16		
5148		105	1/3/16	4/6/16	no pellet ctrl	no treatment	5/6/16	x	x	6/10/16		
5149		103	1/3/16	4/6/16	no pellet ctrl	no treatment	5/6/16	x	x	6/10/16		
5150		101	1/3/16	4/6/16	no pellet ctrl	no treatment	5/6/16	x	x	6/10/16		
5152	25.4	121	1/31/16	4/27/16	erb041	5/17/16	5/20/16	x	x	6/10/16		
5153	26.9	125	2/1/16	4/27/16	erb041	5/17/16	5/20/16	x	x	6/10/16		
5154	24.5	126	2/1/16	4/27/16	erb041	5/17/16	5/20/16	x	x	6/10/16		
5155	21.7	127	2/1/16	4/27/16	erb041	5/17/16	5/20/16	x	x	6/10/16		
5156	27.28	128	2/1/16	5/5/16	erb041	5/17/16	5/20/16	x	x	6/10/16		
5163	24	140	2/11/16	5/5/16	ppt	5/31/16	6/3/16	x	x	6/10/16		
5164	24	139	2/11/16	5/5/16	ppt	5/31/16	6/3/16	x	x	6/10/16		
5165	24	132	2/10/16	5/5/16	e2	5/31/16	6/3/16	x	x	6/10/16		
5166	20	131	2/10/16	5/5/16	ctrl no pellet	5/31/16	6/3/16	x	x	6/10/16		
5167	24	130	2/10/16	5/5/16	e2	5/31/16	6/3/16	x	x	6/10/16		

Figure 4.4 Mice used in this study

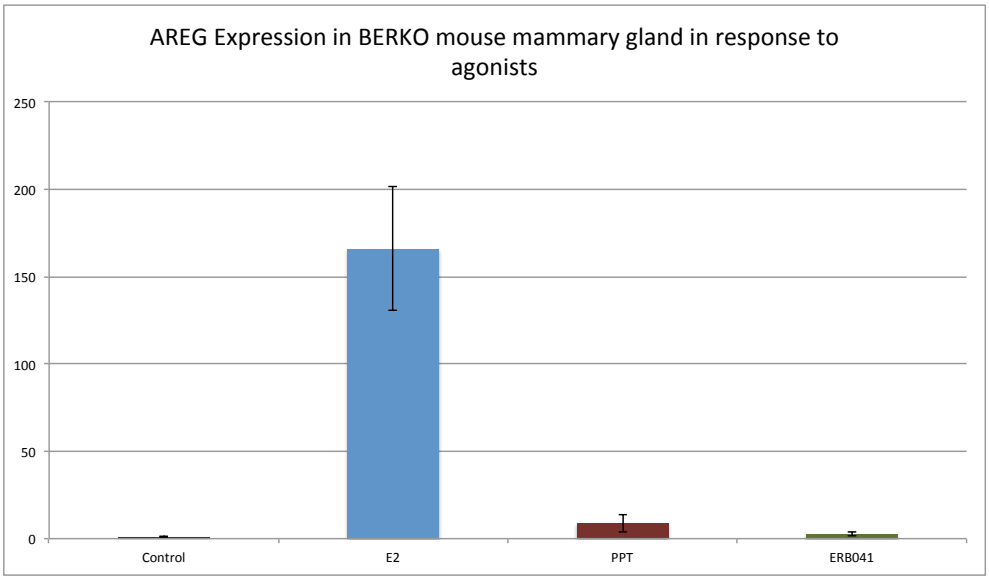

Figure 4.5 RT-qPCR in mouse mammary gland tissue

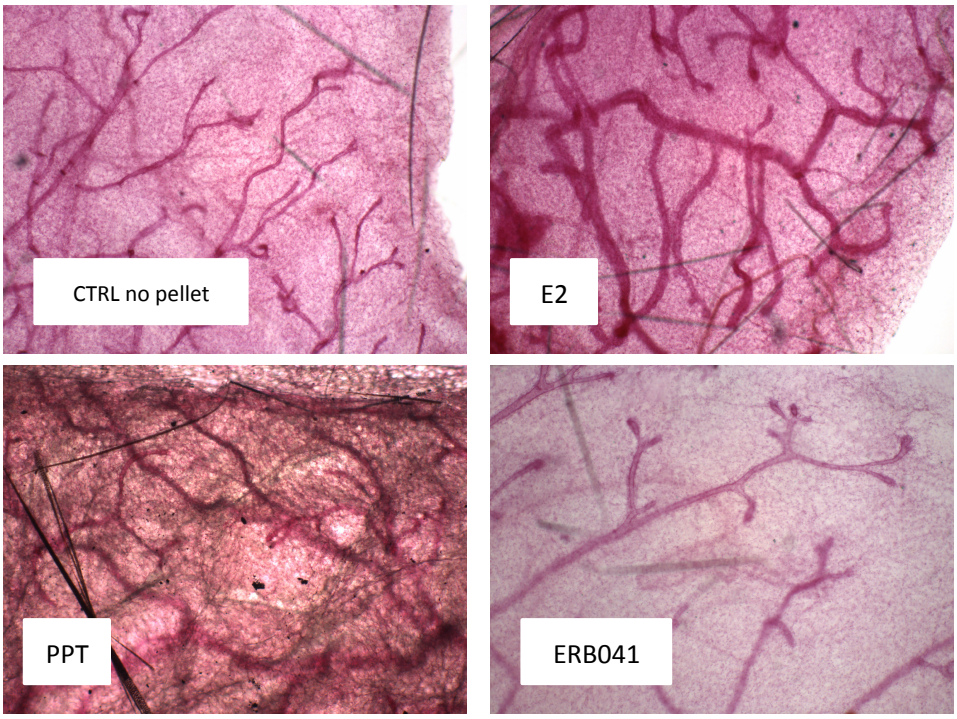

Figure 4.6 Representative βERKO Mouse mammary gland whole mounts harvested 4 days post agonist treatment.

CHAPTER 5

CONCLUSION

Breast cancer cell lines are used as a reference for both agonist induced Estrogen Receptor mediated gene expression as well as elucidating potential therapeutic targets in human breast cancer (Eric, Jennifer, Fernando, & Jordan, 2006; Haldosén, Zhao, & Dahlman-Wright, 2014; Helguero, Faulds, Gustafsson, & Haldosen, 2005). In this study of ER mediated balance between proliferation and growth modulation in human breast cancer, we began by characterizing the Estrogen Receptor status as well as the ratio of ERα to ERβ in six immortalized breast cancer cell lines: T47D, MCF-7, MDA MB 231, MTO ERβ, HCC1937, ZR75-1 and one immortalized "normal" breast cell line 76N tert. We endeavored to link the balance of Estrogen Receptors in these cell lines to ER specific agonist stimulation in order to measure the response through ER protein and gene target expression. For ERα responses we used well-established ERα targets AREG and PR. And to gauge the repressive effects of ERβ stimulation we used CEBPd and TGFβ2. Then, in order to observe in vitro mammary gland responses to ER specific agonists, we utilized an ovariectomized ERβ knockout mouse model.

After determining the baseline expression of ER through qPCR and western blots in our reference breast cancer cell lines, we compared the responses to ER agonists. We were able to make several observations about our reference cell lines based on our results. First, we determined that T47D cells

express both ERα and ERβ by gene and protein expression. Our data also indicates that E2 and PPT upregulated the ERα responsive gene AREG in a dose dependent manner. We observed that ERα expression in T47D cells repressed TGFβ2 and CEBPd expression. Secondly, we found that MCF-7 cells express primarily ERα. In MCF-7 cells, the ER agonist E2 significantly increased the magnitude of AREG expression compared to T47D cells. We believe that in MCF-7 cells, with unopposed ERα expression increases the responsiveness of the cell to E2-mediated proliferation. Next, we observed that HCC1937 cells primarily express ERβ. Also, the HCC1937 cell line expresses high endogenous levels of CEBPd, the potential marker for cell cycle arrest, relative to both ERα and the ERα/ERβ expressing cell lines.

Frustratingly, we observed several results that will have to be resolved in the future. Among the results is that our western blot data indicate that a reliable ERβ antibody remains elusive. Our western blot data is questionable for three reasons. First, the MTO ERβ overexpressing and HCC1937 cell lines had erroneously low ERβ protein expression despite having high and moderate qPCR expression respectively. Secondly, we observed the slow growth pattern of these two cell lines, which is typical of ERβ expressing cells, so we are confident that they are expressing ERβ protein. And finally, the protein band for the “ERβ” antibody observed on the blot was not the correct calculated molecular weight. Among the western blot results we were also surprised to find that we may have contamination of our ZR75-1 cell line. This may have caused the low ERα

expression observed in this traditionally ERα positive cell line. Another result that will need to be addressed is in regard to the in vivo experiments in the βERKO mice. Our results indicate that the low expression of PPT induced AREG observed in qPCR may be due to the thermo instability of PPT for the extended period of time needed for irradiation-based sterilization prior to in vivo testing.

In the future, our lab will choose new cell lines that express ERα primarily, ERβ primarily and express both ERα and ERβ to test responses to Estrogen Receptor specific agonists. We will also retry PPT treatments in βERKO mice as well as in wild type mice. We will also run qPCR on the harvested βERKO mammary tissue in order to determine the expression of CEBPd and TGFβ2.

To conclude, ERα and ERβ are just parts of the dynamic tension that determines breast cancer cell proliferation and cell cycle arrest. Building upon the results presented here, we hope to improve our understanding of the Estrogen Receptor status of our reference cell lines for future use.

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